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Thy-1(CD90) expression in dental pulp stem cells

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Thy-1 (CD90) Expression in Dental Pulp Stem Cells

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A Thesis Submitted for the Degree of Doctor of Philosophy at the
University of London

2012

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Abstract

The search for mesenchymal stem cells (MSCs) in adult tissues had led to the discovery of stem cells in many tissues in the body, including teeth. A better understanding of the biology of dental stem cell populations is a pre-requisite to allow their future use for clinical purposes.

To understand the biology of stem cell niches in the dental pulp, new genes expressed in dental tissues that could serve as markers of dental stem cells were investigated. The mouse incisor differs from mouse molars as well as human teeth in that it erupts continuously throughout the life of the animal. This continuous growth and eruption is related to the presence of the stem cells at the cervical end of the incisor to replace those lost from the incisor tip producing enamel and dentin of the erupting tooth.

From a mouse genome microarray using incisor body and cervical pulp cells, several genes were found to be up-regulated in the cervical area which has a MSC niche that provides a source of cells to replace those lost during continuous growth. Thy-1 (CD90) was selected as a stem cell marker to explore mesenchymal stem cell niches in murine dental pulp tissue. Protein expression was confirmed by flow cytometry and Thy-1 expression in cell culture was investigated. Its expression declined gradually with increasing cell passages. Whole mount *in situ* hybridization at different developmental stages showed that Thy-1 was selectively expressed in the cervical area of incisors, where the MSC niche is located. In order to identify the function of this gene, genetic lineage tracing was used. Thy-1Cre expressing transgenic mice were crossed with Rosa26R reporter mice to follow the fates of Thy-1 expressing cells. Thy-1⁺ cells contributed to odontoblasts and pulp cells during incisor growth. However not all odontoblasts and pulp cells were Thy-1⁺ suggesting a complex MSC niche. Sonic Hedgehog (Shh) is the principle hedgehog gene in adult mouse incisors and cells responsive to it are stem cells inside the mouse incisor. Using transgenic mice where Shh signaling was modulated, Thy-1 expression was shown to be regulated *in vivo* by Shh in adult mouse incisor. Its expression was increased in Ptc-1^{fl/fl};CreERTm mice and was decreased in Polaris^{fl/fl};CreERTm mice. Therefore, Thy-1 is a marker for stem cell subpopulation in mouse incisor dental pulp and it is regulated by Shh.

Acknowledgement

First of all, Praise is to **ALLAH** who gave me life and strength to go through this project. I am indebted and grateful to many people for helping me in carrying out this work.

I cannot mention all of them, but the following are a few :

I would like to express my sincere gratitude to my supervisor, **Professor Paul Sharpe**, for his patience, vast knowledge, practical suggestions and help from the very beginning till the end of the project. I am highly grateful to my second supervisor, **Dr Agi Grigoriadis** for his great contribution and kind assistance throughout my study.

I am immensely grateful for **Dr Isabelle Miletich** for her detailed and constructive comments in the preparation for writing this thesis. I also wish to thank **Dr Shelly Abraham**, for her valuable feedback during the writing of this thesis. Special thanks go to **Dr. Christopher Healy** for providing technical support throughout my work in CFD. I can hardly find the enough words to thank **Dr Andrea Mantesso** for all her assistance in writing my thesis and her readiness to listen and answer all my questions. Sincere thanks go to **Dr Wenny** and **Dr Ana Angelova** for their help in writing my thesis, their continuous support and encouragement throughout my PhD project.

A word of gratitude is extended to all former and current **CFD2 members: Atsushi, Miko, Katsu, Shelly, Yvonne, Long Long, Abbas, Nisha, Jifan, Sarah, and N'first**, and many more people in CFD; all of them have taken part in my inquiry at various stages/times. Special thanks go to **Dr Simone** and **Silvia** from Brazil for being such wonderful companions in lab and coffee time as well. My warm thanks are also extended to **Angela, Sharon** and **Martin** for their kind assistance whenever I needed it.

I forever owe **my husband, Ahmed** and **my lovely cute kids, Abdalla** and **Omar** for their patience and constant support throughout my study abroad in the UK. Big thanks to them for sacrificing their rights to give me more time in the lab. All love and blessing go to my big **beloved family members** who passed away without being able to say 'Goodbye'. May you lie in peace, and see you in heaven. Very warm thanks must go to **my respectful parents** whose encouragement and support have never been missing, **I love you all**. The financial support of the **Egyptian Government** and **The Faculty of Dentistry, Tanta University** are also gratefully acknowledged.

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List of Abbreviations

ASC	Adult stem cells
APC	Allophycocyanin
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BrdU	5-bromo-2'-deoxyuridine
Body	Pulp body
Crabp-1	Cellular Retinoid Acid Binding Protein-1
CL	Cervical Loop
c-Myc	c-myc myelocytomatosis viral oncogene homolog
CVADR	Coxsackie Virus and Adenovirus Receptor
Cyc	Cyclopmaine
DFPCs	Dental follicle progenitor stem cells
DEPC	Diethyl pyrocarbonate
Dil	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMSO	Dimethyl sulfoxide
DLX5	Distal-less homeobox 5
DDT	DL-Dithiothreitol
DPBS	Dulbecco's Phosphate Buffered Saline
Est-1	E Twenty Six 1
ES	Embryonic Stem cells
ER	Estrogen Receptor
ECS	Endothelial Cells
EGFP	Enhanced Green Fluorescent Protein
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
Fak	Focal adhesion kinase F-actin
FC	Fold Change

GliAct	Gli-1 activator
Gli3Rep	Gli-3 repressor
Gli	Glioblastoma
Hsp90	Heat shock protein 90
h	Human
Hus-1	Hydroxy Urea Sensitive-1
HA/TCP	Hydroxy-Apatite/Tri-Calcium-Phosphate
IL-Ira	IL-1 receptor antagonist
iPS	induced pluripotent stem cells
IL	interleukin
ISCT	International Society for Cellular Therapy
IFT	intraflagellar transport proteins
Klf4	Krueppel-like factor 4
LBD	ligand-binding domain
LTP	Long-term potentiation
LB	Luria-Bertani
MACS	Magnetic Activated Cell Sorting
MSC	Mesenchymal stem cells
m	Mouse
MPPs	multipotent progenitors
NBT	4-Nitro blue tetrazolium chloride
NPC	Nasopharyngeal carcinoma
NG2	Neural/Glial Antigen 2
NF	Neurofilament
Neurog3	Neurogenin 3
NMDA	N-methyl-D-aspartate
NOD/SCID	Non-Obese, Diabetic/Severe, Combined Immunodeficient
Orpk	Oak Ridge Polycystic Kidney

OCT3/4	octamer-binding transcription factor 3/4
OPT	Optical Projection Tomography
mafa	Pancreatic beta-cell-specific transcriptional activator
Pdx-1	Pancreatic and duodenal homeobox 1
PFA	Paraformaldehyde
Ptc-1	Patched-1
PDLSCs	Periodontal ligament stem cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PDGER β	Platelet-derived growth factor receptor-beta
ROS	Reactive oxygen species
Rcf	Relative centrifugal force
RA	Retinoic Acid
Smo	Smoothened
SAG	Smoothened Agonist
SDS	Sodium dodecyl sulphate
Shh	Sonic hedgehog
SRX	Sex determining region Y
SCAP	Stem cells from apical papilla
SHED	Stem cells from human exfoliated deciduous teeth
Tam	Tamoxifen
TCR	T-cell receptor
Thbs-1	Thrombospondin 1
TGF β	Transforming growth factor- β
TEA	Triethanolamine
TuJ-1	Tubulin III
TNF	Tumor Necrosis
Znfn1a1	Zinc Finger protein, subfamily 1A, 1

Chapter 1 General Introduction

1.1 Stem cells

Stem cells are clonogenic cells that have two main features, the ability to differentiate into multiple mature cell types and the ability to self-renew that allows them to sustain tissue development and maintenance (Weissman, 2000). Stem cells reside in highly regulated micro-environments called niches, which allow them to maintain a balance between self-renewal and differentiation. These micro-environments are maintained by a constant dialogue between the stem cells and the surrounding differentiated cells. The niche provides a shelter for the stem cells from differentiation stimuli, apoptotic stimuli and any other stimuli that might challenge stem cell stores. The niche must also protect stem cells from overproduction, which if not properly controlled may lead to cancer (Moore and Lemischka, 2006).

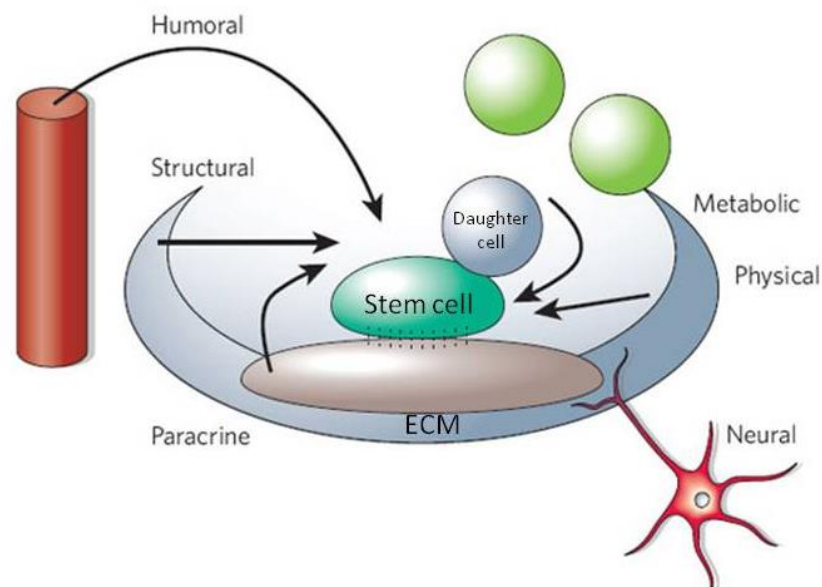


Figure 1-1: Schematic of a generic somatic stem cell niche: showing the various elements that participate in regulating the fate of the resident stem cell. The distance from the ultimate stem cell position may determine the relative strength of each of these signals and thus contribute to the differentiation of the daughter cell. *ECM* extracellular matrix. (Modified from (Scadden, 2006).

Stem cells can be classified as totipotent, pluripotent, multipotent or unipotent, according to their ability to differentiate. Totipotent stem cells are those that can be implanted through the blastocyst into the uterus of a living animal and give rise to a complete organism. Pluripotent stem cells are those that can give rise to every cell of an organism except its extra-embryonic tissues, such as the placenta. Finally, multipotent stem cells only generate specific cell lineages, whilst unipotent stem/progenitor cells have the capacity to differentiate into only one cell type (Figure 1-2).

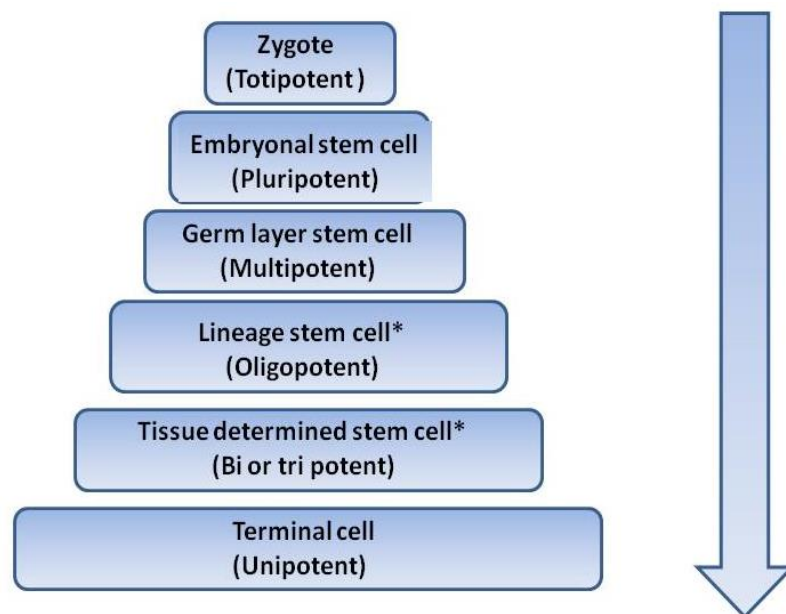


Figure 1-2: Stem cell hierarchy during differentiation. At each stage, differential potential decreases and specialization increases. (*)These are also called transit-amplifying cells (Sell, 2004).

Developmentally, there are two main categories of stem cells: embryonic stem cells (ES) and postnatal or adult stem cells (ASC) (McKay, 2000, Leeb et al., 2010).

1.1.1 Embryonic stem cells

ES cells are stem cells derived from the inner cell mass of an early pre-implantation stage embryo known as a blastocyst (Evans and Kaufman, 1981). Although a number of species-related differences have been documented (e.g., growth characteristics, doubling

time, cell surface markers, signalling pathways, and cultivation conditions), these cells are pluripotent (Yamanaka et al., 2008). Mouse (m) ES cells can generate a fully viable embryo, including all cell types. Human (h)ES cells contribute to all three germ layers in teratomas (a benign tumor typically containing a mixture of many differentiated or partially differentiated cell types) induced in nude mice (Nussbaum et al., 2007). ES-cell lines have an almost unlimited capacity to proliferate and differentiate *in vitro*. Consequently, these pluripotent stem cells are able to generate all other multipotent and unipotent stem cells found in the embryo (Wobus and Boheler, 2005). Although ES cells are a useful tool for regenerative medicine and tissue engineering, they have a high neoplastic potential. ES cells display many features characteristic of cancer cells (Burdon et al., 1999) including unlimited proliferative capacity (Suda et al., 1987) clonal propagation and lack of both contact inhibition and anchorage dependence. Moreover, their use is controversial and is surrounded by ethical and legal issues (Takahashi and Yamanaka, 2006a).

1.1.2 Adult stem cells

As development proceeds, except for germ cells, pluripotent embryonic stem cells disappear. In the adult organism, although most of the cells are committed, most tissues have been shown to contain a small sub-population of cells, known as adult stem cells (ASC) with the innate ability to maintain a stem cell pool by self-replication and generate more committed progenitors through differentiation along multiple lineages (Weissman, 2000). Such cells usually remain quiescent within the adult tissue; however, they may respond to tissue injury and play an integral role in the tissue repair processes (Fuchs et al., 2004). Moreover, their high potential for expansion '*in vitro*' and the immunomodulatory properties of mesenchymal cells make them of great interest in the scientific community (Leeb et al., 2010). In the past six years, a new type of adult stem

cells has emerged, named induced pluripotent stem cells (iPS). iPS are somatic cells that are genetically "reprogrammed" in the laboratory environment by different methods such as lentoviral, retroviral, plasmid, adenoviral or modified mRNA (Robinton and Daley, 2012) to introduce four factors: octamer-binding transcription factor 3/4 (Oct3/4), SRY (sex determining region Y) -box 2 also known as (SOX2), Krueppel-like factor 4 (Klf 4), and c-myc myelocytomatosis viral oncogene homolog (c-Myc) into pluripotent stem cell-like states (Takahashi and Yamanaka, 2006b, Takahashi et al., 2007). In another study, iPS cells were generated from human fibroblasts using Oct4, Sox2, Nanog, and Lin28 (Yu et al., 2007a). Unlike ES cells, their use in research and medical applications is less controversial because they can be harvested without destroying an embryo. Nevertheless, although the four reprogramming factors were different in these two studies, all of them were transferred into the cells by means of retrovirus gene transfer, which holds some risk of causing insertion mutations. In addition, since some of the reprogramming factors are oncogenes as well, the risk of tumour induction is another potential limitation to be considered.

A recent breakthrough study succeeded in directly reprogramming pancreatic exocrine cells to insulin producing beta cells, using a combination of three transcription factors: Neurogenin 3 (Neurog3), (Pancreatic and duodenal homeobox 1 (Pdx1) and Pancreatic beta-cell-specific transcriptional activator (mafa) (Zhou et al., 2008). This method is more advanced than iPS cells, as they used a strategy for cellular reprogramming using defined factors in an adult organ, suggesting a general paradigm for directing cell reprogramming without reversion to a pluripotent stem cell state.

1.2 Mesenchymal stem cells

Mesenchymal stem cells (MSC) constitute a sub-population of ASC. Classically, MSCs were defined as non-hematopoietic cells that reside in the Bone Marrow (He et al.), together with hematopoietic stem cells. They were first described by Friedenstein et al. in 1976, as clonal, plastic-adherent cells being a source of osteoblastic, adipogenic and chondrogenic cell lines (Friedenstein et al., 1976). The interest in MSCs rapidly grew with the expanding knowledge about their exceptional characteristics, which led to their discovery in other species, including humans (Castro-Malaspina et al., 1980) and mice (Sung et al., 2008), and also in many tissues other than bone marrow, including peripheral blood (Zvaifler et al., 2000), cord blood (Erices et al., 2000), adipose tissue (Zuk et al., 2002), amniotic fluid (In 't Anker et al., 2003), compact bone (Guo et al., 2006), periosteum (Nakahara et al., 1991), synovial membrane (De Bari et al., 2003) and synovial fluid (Jones et al., 2004), articular cartilage (Dowthwaite et al., 2004) and foetal tissues (Miao et al., 2006).

The name MSC now refers to mesenchymal stem cells found in tissues other than BM. Due to its inaccuracy, this term has caused frequent misconceptions, as it refers to both *in vivo* and *in vitro* expanded progeny. Consequently, the International Society for Cellular Therapy (ISCT) has stated that the current data are insufficient to classify un-fractionated plastic-adherent marrow cells as stem cells (Horwitz et al., 2005). Instead, the ISCT suggests the use of the term “multipotent mesenchymal stromal cell” to indicate these unique properties without ascribing homogeneity or stem cell activity; while the term “mesenchymal stem cells” should be reserved for long-term self-renewing cells that are capable of differentiating into specific, multiple cell types *in vivo*. For both of these cell populations, the acronym MSC may be used. Moreover they propose three criteria to define MSC. First, MSC must be plastic-adherent when maintained in standard culture

conditions using tissue culture flasks. Secondly, 95% or more of the MSC population must express CD105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack expression (≤ 2 positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. Thirdly, the cells must be able to differentiate into osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions (Horwitz et al., 2005). Details about these markers are summarized in table (1-1).

Table 1-1: Details of other functions of MSC CD markers.

MSC marker	Function
CD105 (Endoglin)	It is a proliferation-associated and hypoxia-inducible protein abundantly expressed in endothelial cells (EC). It is a receptor for transforming growth factor (TGF) β 1 and β 3 and modulates TGF- β signaling by interacting with TGF- β receptors I and/or II (DUFF et al., 2003)
CD73	Cell surface enzyme expressed on many cell types including subsets of lymphocytes, endothelial cells and epithelial cells. During inflammation, it converts AMP to extracellular adenosine Which has potent immunosuppressive effects (Mills et al., 2008).
CD45 (Common leukocyte antigen)	It is a protein tyrosine phosphatase (PTP) located in hematopoietic cells except ethrocytes and platelets. CD45 is a protein that has several isoforms ,the specified expression of the CD45 isoforms can be seen in the various stages of differentiation of normal hematopoietic cells . CD45 is uniformly distributed in plasma membrane and enrich regions of T cell and B cell contact (SLOAN, 1997).
CD34	It is a member of a family of single-pass transmembrane sialomucin proteins that show expression on early hematopoietic and vascular-associated tissue.

	It functions as a cell-cell adhesion factor. It may also mediate the attachment of stem cells to bone marrow extracellular matrix or directly to stromal cells (Simmons et al., 1992).
CD14 (Human gene)	<p>Its protein is a component of the innate immune system. CD14 exists in two forms. Either it is anchored into the membrane by a glycosylphosphatidylinositol tail (mCD14) or a soluble form (sCD14).</p> <p>It is expressed mainly by macrophages, by neutrophil granulocytes and dendritic cells. sCD14 is also present in human milk, where it is believed to regulate microbial growth in the infant gut.</p> <p>CD14 acts as a co-receptor for the detection of bacterial lipopolysaccharide (LPS) to induce endocytosis (Zanoni et al., 2011).</p>
CD11b (Human gene)	<p>It is Expressed on the surface of many leukocytes including monocytes, neutrophils, natural killer cells, granulocytes and macrophages, as well as on 8% of spleen cells and 44% of bone marrow cells</p> <p>It Regulates leukocyte adhesion and migration to mediate the inflammatory response and cellular adhesion (Han et al., 2010)</p>
CD79a (mb-1) (Human gene)	It Forms a dimer associated with membrane-bound immunoglobulin in B-cells together with the related CD79b protein. This dimer is closely associated with the B-cell antigen receptor and enables the cell to respond to the presence of antigens on its surface
CD19	<p>It is expressed on follicular dendritic cells and B cells.</p> <p>It primarily acts as a B cell co-receptor in conjunction with CD21 and CD81 (Tedder and Isaacs, 1989).</p>

Therefore, the definition of MSC relies solely on the analysis of *in vitro* culture of expanded cell populations. It is important to keep in mind that the MSC phenotype and abilities can be different in *in vivo* and *in vitro* settings due to their removal from their natural environment, as well as the use of chemical and physical growth conditions that might alter their characteristics (Augello et al., 2010). For example, MSCs are known to undergo phenotypic rearrangements during *ex vivo* manipulations, losing expression of some markers while acquiring expression of new ones (Jones et al., 2002). Moreover, using the same cell strain, multipotency of MSCs assessed by *in vitro* differentiation assays correlated poorly with results of *in vivo* differentiation assays (Bianco et al., 2008). Furthermore, multipotency cannot be determined with assays conducted on non-clonal cell strains in culture. Indeed, *in vitro* generation of alizarin red deposits (osteogenesis), oil red O-stainable cells (adipogenesis), and alcian blue-stainable matrix (chondrogenesis) in parallel cultures of non-clonal MSC strains, does not predict multipotency as commonly assumed in the literature as it is not necessarily for cells to differentiate *in vivo* as they do *in vitro* (Gronthos et al., 2000a). Claims for *in vivo* differentiation into other cell types are equally controversial, as BM mesenchymal stromal cell cultures have been shown to contribute to many tissues following transplantation through fusion with endogenous cells and not through differentiation into mature cell types (Alvarez-Dolado et al., 2003).

A key task for assessing the function of MSCs *in vivo* is to define their localisation *in situ* in organs. Efforts to track the identity of tissue-resident MSCs had consistently suggested that these cells lie adjacent to blood vessels as pericytes (Corselli et al., 2010). Pericytes, also known as Rouget cells or mural cells, are defined by their perivascular location and morphology. They are embedded in basal membranes enveloping the capillary tubes and have prominent nuclei and relatively limited perinuclear cytoplasm, from which they

extend the processes that directly contact and communicate with the endothelial cells (Edelman et al., 2006). Pericytes may display MSC-like features, as cells expressing mesenchymal stromal cell markers such as CD146 and CD90 were found in blood vessel walls in human bone marrow and dental pulp (Doherty et al., 1998, Shi and Gronthos, 2003). Furthermore, MSC-like cultures were generated from cells expressing pericyte-specific markers (Schwab and Gargett, 2007) (Figure 1-3).

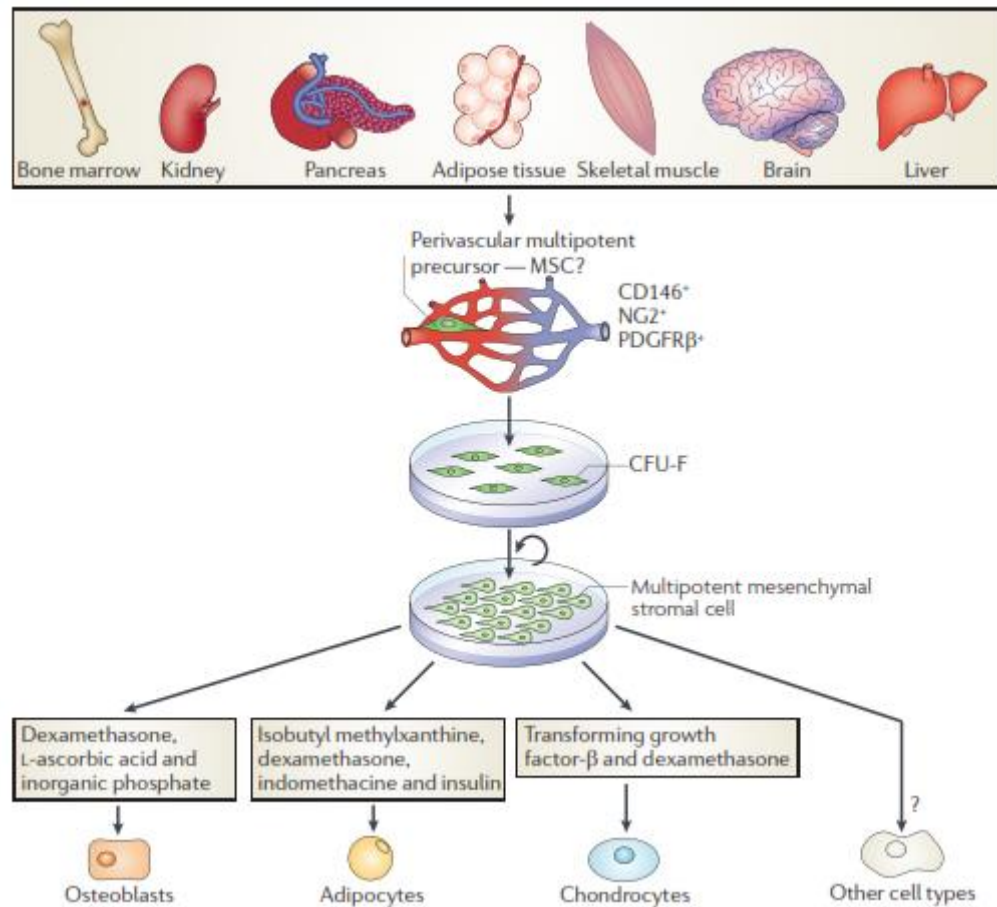


Figure 1-3: MSCs and multipotent mesenchymal stromal cells. proposed multipotent progenitor cell population, that resides in the proximity of blood vessels in tissues studied so far, has been shown to express pericyte-specific markers (CD146, NG2 (also known as CSPG4) and platelet-derived growth factor receptor-β (PDGFRβ)). When cultured at appropriate cell densities, colonies derived from single colony forming unit-fibroblast (CFU-Fs) can be isolated and expanded after multiple passages *in vitro* (indicated by the curved arrow) without losing their multipotent mesenchymal capacity. These cultured cells, classically referred to as mesenchymal stem cells (MSCs), are now termed multipotent mesenchymal stromal cells. The hallmark that defines mesenchymal stromal cells is their ability to differentiate into osteoblasts, adipocytes and chondrocytes when placed under inductive stimuli. Differentiation into multiple non-mesenchymal mature cell types (such as neural cells) has been reported but remains a matter of debate (Nombela-Arrieta et al., 2011a).

Pericyte-derived cultures are similar to mesenchymal stromal cell cultures in terms of morphology and cell surface antigen expression, and can be induced to differentiate into osteoblasts, chondrocytes, adipocytes, smooth muscle cells and myocytes under appropriate conditions (Farrington-Rock et al., 2004, Doherty et al., 1998, Collett and Canfield, 2005). A combination of markers, such as NG2, CD146 and PDGFRβ appears to specifically label pericytes in a range of human organs, including fetal and adult skin, pancreas, heart, brain, lung, bone marrow and placenta (Crisan et al., 2008a). They also

displayed tri-lineage potential *in vitro* and osteogenic potential *in vivo* (Crisan et al., 2008a). Collectively, these results strongly suggest that the precursors of cultured mesenchymal stromal cells preferentially reside close to blood vessels *in vivo*, a trait that is relevant to all multipotent stem or progenitor cells present in adult tissues.

Nevertheless, it is important to note that the terms pericyte and MSC are not equivalent or interchangeable. Although the word pericyte etymologically refers to cells surrounding the blood vessels, the term pericyte strictly refers to cells adjacent to capillaries and post-capillary venules (Hirschi and D'Amore, 1996). However, multipotent MSC-like precursors have been isolated from the walls of other vascular types, including arteries and veins (Tintut et al., 2003, Hoshino et al., 2008). Furthermore, because pericytes show an extensive tissue distribution along diverse microvascular beds and have many proposed functions (including vessel stabilisation, phagocytosis and regulation of vascular integrity (Hirschi and D'Amore, 1996), it is likely that functionally heterogeneous, non-equivalent cell subsets are included under the vague term of pericyte. Thus, despite being peri-vascular, not all MSCs can be referred to as pericytes, and not all pericytes exhibit MSC properties.

One of the most remarkable and unforeseen aspects of mesenchymal stromal cells pertains to their immunomodulatory activity (Bernardo et al., 2009). *In vitro*, mesenchymal stromal cells inhibit T cell activation, dendritic cell differentiation, B cell proliferation, and impair the cytolytic potential of natural killer cells. These effects are partially explained by the ability of mesenchymal stromal cells to secrete a vast array of soluble mediators with immunomodulatory properties such as interleukin10 (IL-10), prostaglandin E2, nitric oxide and transforming growth factor- β (TGF β) (Meirelles Lda et

al., 2009). Nevertheless, these immunomodulatory effects require, at least in part, direct cell to cell contact (Sotiropoulou et al., 2006).

Immunomodulation *in vitro* and *in vivo* has been reported exclusively for mesenchymal stromal cells. In addition, MSCs are able to induce a similar degree of inhibition in the presence of both autologous and allogeneic responder cells, thus supporting the concept that MSCs can be considered universal suppressors (Le Blanc et al., 2003, Krampera et al., 2003). On the other hand, MSCs were shown to be effectively lysed by autologous and allogeneic IL2 activated NK cells (Spaggiari et al., 2006, Barry and Murphy, 2004). Moreover, when infused into MHC mismatched mice, MSCs could be lysed by cytotoxic T lymphocytes, resulting in their rejection (Nauta et al., 2006). In fact, studies of the mechanism by which MSCs display their immunosuppressive effect are largely restricted to *in vitro* studies and no evidence exists to date to suggest that such regulatory properties can be ascribed to MSCs *in vivo*. However, given that the BM is one of the sites where adaptive immune responses are generated, and that BM-resident MSCs share perisinusoidal locations with dendritic cells and circulating B cells, it seems plausible that MSC-immune cell interactions may be of physiological relevance (Nombela-Arrieta et al., 2011b). Recently, Oral Mucosal lamina propria progenitor cells (OMLP-PCs) showed their immunosuppressive effect through a dose and HLA-II independent mechanism by release of soluble immunosuppressive factors such as indoleamine 2,3-dioxygenase (IDO) (Davies et al., 2012).

1.3 Dental stem cells

Dental stem cells have been identified as candidates for tooth tissue engineering because of their multipotent differentiation ability. These cells also provide an alternative for use in regenerative medicine, since they can be used to regenerate not only dental tissues, but also non-dental tissues such as bone and nerves.

Several types of dental stem cells have been isolated from teeth, including dental pulp stem cells (DPSCs) (Gronthos et al., 2000a), stem cells from human exfoliated deciduous teeth (SHEDs) (Miura et al., 2003), periodontal ligament stem cells (PDLSCs) (Seo et al., 2004), dental follicle progenitor stem cells (DFPCs), (Morsczeck et al., 2005) and stem cells from apical papilla (SCAPs) (Sonoyama et al., 2006). It is however important to consider that although different types of dental tissue-derived MSCs share several common characteristics, they present significant heterogeneity, in the form of multiple phenotypic differences, which most probably reflect distinct functional properties (Bianco et al., 2008). This is complicated by the fact that researchers have used different methods to isolate and culture dental MSCs and evaluate their differentiation potential.

1.3.1 Stem cells from the apical papilla (SCAP)

SCAPs are dental stem cells that have been discovered in the apical papilla of human immature permanent teeth (Sonoyama et al., 2006). The apical papilla contains precursor tissue for the radicular pulp. SCAPs, obtained by explant cultures or enzymatic digestion of apical pulp tissue, express mesenchymal markers such as CD13, CD24, CD29, CD44, CD73, CD90, CD105, CD106 and CD146 and do not express CD18, CD34, CD45, or CD150 (Ding et al., 2010b). Stem cells from the apical papilla have the capacity to undergo osteo/dentinogenic, neurogenic, and adipogenic differentiation (Sonoyama et al., 2006). SCAPs display osteo/dentinogenic markers and growth factor receptors similar to

DPSCs, but these markers are expressed at lower levels in SCAPs than in DPSCs (Sonoyama et al., 2008). To date the myogenic and chondrogenic differentiation potential of SCAPs has not been determined (Sonoyama et al., 2006, Abe et al., 2007). Furthermore, in several reported cases of apexogenesis in an infected immature tooth with periradicular periodontitis or an abscess, SCAPs had the ability to induce root formation (Huang et al., 2009, Huang et al., 2008, Friedlander et al., 2009, Chueh and Huang, 2006). When *ex vivo* expanded human SCAPs were transplanted subcutaneously into the dorsal surface of 10-week old immunocompromised mice with Hydroxy-Apatite/Tri-Calcium-Phosphate (HA/TCP) as a carrier, typical dentine structure was generated. Dentine-forming cells were recognized by antibodies directed against human mitochondria, suggesting that SCAP-derived human cells from the donor contributed to dentine formation (Sonoyama et al., 2006). In a comparison between immature root papilla stem cells (iRPSCs) and mature root pulp stem cells (mRPSCs) at the root-forming stage revealed that, iRPSCs presented stronger dentinogenesis but weaker osteogenesis than did mRPSCs, suggesting that the dentinogenic competence of root mesenchymal stem cells decreases, whereas their osteogenic potential increases following the maturation of the tooth root (Lei et al., 2011).

1.3.2 Periodontal ligaments stem cells (PDLSCs)

Seo et al. (2004) suggested that the human periodontal ligament (PDL) contains a population of postnatal multipotent stem cells that can be isolated and expanded *in vitro* using explant cultures or enzymatic digestion. PDLSCs express MSC markers such as CD10, CD13, CD29, CD44, CD59, CD73, CD90 and CD105, and do not express CD14, CD34, CD45 and HLA-DR (Feng et al., 2010, Wada et al., 2009). PDLSCs have the capacity to differentiate into cells similar to cementoblasts and collagen-forming cells. Formation of calcified nodules is less prominent than that observed with DPSCs and

SHEDs. Furthermore, PDLSCs have the ability to differentiate *in vitro* into adipogenic, osteogenic and chondrogenic cells (Gay et al., 2007, Xu et al., 2009). *In vivo*, PDLSCs can differentiate into functional cementoblasts, when transplanted subcutaneously on the dorsum of immunocompromised mice, and can form collagen fibres embedded in the cementum-like tissue, suggesting that these cells have the potential to regenerate the cementum and PDL *in vivo* (Seo et al., 2004). The same group investigated whether the PDLSCs were able to contribute to periodontal tissue repair by transplanting these cells into surgically created periodontal defects in the molar area of the mandible of immunocompromised rats. Transplanted PDLSCs were integrated into the jaw tissue of two of the six rats operated and were found at the surfaces of the alveolar bone and teeth. The investigators concluded that PDLSCs play a role in the repair of the periodontal tissue and have the potential to regenerate damaged PDL (Seo et al., 2004). In addition, PDLSCs transplanted into the tooth sockets of the mandible of a mini-pig with HA/TCP as a carrier, formed an artificial bio-root encircled with PDL tissue (Sonoyama et al., 2006).

1.3.3 Stem cells from exfoliated deciduous teeth (SHEDs)

Miura et al. (2003) first reported that stem cells could be obtained from human deciduous teeth. These multipotent cells are DPSCs that are derived from dental pulp explants or obtained by digestion of dental pulp tissue (Miura et al., 2003). The morphology of SHEDs is small spindle-shaped fibroblastoid cells, which subsequently form large dense colonies (Pivoriunas et al., 2010) similar to that of DPSCs, SCAPs and DFPSs (Rodríguez-Lozano et al., 2011). SHEDs have a higher proliferation rate than bone marrow mesenchymal stem cells (BMMSCs) and DPSCs and express Oct4, CD13, CD29, CD44, CD73, CD90, CD105, CD146 and CD166, but do not express CD14, CD34, or CD45 (Huang et al., 2009, Pivoriunas et al., 2010). SHED cells are capable of

differentiating into a variety of cells, such as neural cells, osteoblasts, chondrocytes, adipocytes and myocytes (Miura et al., 2003, Wang et al., 2010). After subcutaneous transplantation on the dorsum of immune-compromised mice, SHEDs formed ectopic dentine-like tissue, but were unable to generate a dentine/pulp-like complex (Pivoriunas et al., 2010). These results suggest that SHEDs can differentiate into odontoblasts *in vivo*. Additionally, the mineralized tissue generated by SHEDs in the pulp space of tooth-slice scaffolds had morphological features of dentine, including the presence of dentinal tubules and pre dentine, which distinguishes it from osteoid tissue (Sakai et al., 2010). SHEDs are also capable of repairing critical-size parietal defects in immune-compromised mice; however, the bone generated by these cells lacks haematopoietic marrow elements (Miura et al., 2003).

1.3.4 Stem cells from the dental follicle (DFPCs)

The dental follicle is a mesenchymal tissue that surrounds the developing tooth germ and contains precursors of the periodontium. These cells form low numbers of adherent clonogenic colonies when released from the tissue by enzymatic digestion and can be maintained in culture for at least 15 passages (Morsczeck et al., 2005, Yao et al., 2008). DFPCs express CD10, CD13, CD29, CD44, CD53, CD59, CD73, CD90 and CD105, and do not express CD34, CD45, or HLA-DR (Lindroos et al., 2008, Yagyuu et al., 2010, Huang et al., 2009). DFPCs have the ability to differentiate into osteoblasts, cementoblasts, chondrocytes and adipocytes when grown in appropriate media (Yao et al., 2008). *In vitro* findings suggest that DPSCs have greater hard tissue-forming potential than DFPCs. This might be explained by the developmental stage of the tooth germs from which these cells were derived. They were isolated from the crown forming stage in which dentinogenesis can be detected in certain areas of the dental papilla, whereas cementogenesis couldn't be detected in the dental follicle. Thus, there are more

cells that have already committed to hard tissue-forming cells in dental papillae than there are in dental follicles at this stage (Yagyuu et al., 2010). Immortalized dental follicle cells are able to re-create a new periodontal ligament (PDL) after *in vivo* implantation, characterized by expressed periostin, Scleraxis (Scx) and type XII collagen and the fibrillar assembly of type I collagen (Yokoi et al., 2007). However, hard tissues such as dentine, cementum, or bone have not been identified after transplantation of these cells into immunocompromised mice (Yagyuu et al., 2010).

1.3.5 Dental pulp stem cells

1.3.5.1 Dental pulp

Dental pulp is a fibrous connective tissue that occupies the central portion of the tooth enclosed by dentine. Structurally, the dental pulp is composed of cellular, vascular, neuronal and matrix components that contain fibrous components e.g. collagen type I, II and non-fibrous components e.g. proteoglycans (Nanci, 2007). Histologically, the dental pulp consists of four layers from outside to inside: odontoblasts and subodontoblastic cells (Goldberg and Smith, 2004), cell-free zone rich in collagen fibres and poor in cells, cell rich zone richly populated with stem/ progenitor cells (Jo et al., 2007) and core of the pulp containing the vascular plexus and nerves (Figure 1-4).

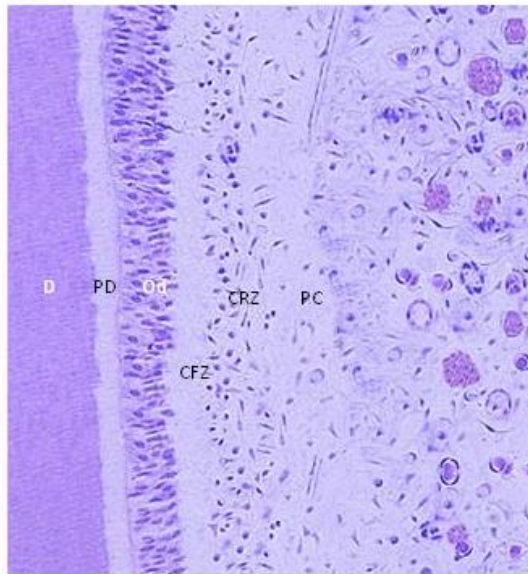


Figure 1-4: Histological view of a longitudinal section through the dental pulp showing the multiple pulp zones. Odontoblastic cell zone (OD), cell-free zone (CFZ), cell-rich zone (CRZ) and pulp core (PC). Dentine (D), predentine (PD) (Melfi and Alley, 2000).

1.3.5.2 Development and origin of dental pulp cells

After the migration of cranial neural crest (CNC) cells into the head and neck structures, a series of sequential and reciprocal interactions between the CNC-derived mesenchyme and the oral epithelium leads to tooth development (Jernvall and Thesleff, 2000). Tooth development is characterized by a series of different stages that are named according to the shape of the dental epithelium (Figure 1-4). The first morphological sign of tooth development is observed as a thickening of the oral epithelium, which subsequently invaginates into the underlying mesenchymal tissue and forms a bud-shaped epithelium. At the same time, the mesenchyme surrounding the epithelial bud condenses. The epithelium then expands and folds to form a cap. The condensed mesenchyme partly surrounded by the cap-shaped epithelium is the dental papilla, which will give rise to the dentine-producing odontoblasts and the dental pulp. The outermost cells of the dental papilla and those surrounding the epithelial dental compartment proliferate to form the

dental follicle or dental sac. When the roots of the teeth develop, the dental sac will produce the cells forming the tooth-supporting tissues, which include the cementum, the periodontal ligament and the alveolar bone (Nanci, 2007). Chai, et al. (2000), used a genetic marker to follow the progeny of CNC cells in the mouse. They demonstrated that in the developing tooth germ, the CNC-derived ectomesenchyme contributes to the condensed dental ectomesenchyme during the bud stage, and subsequently to the formation of the dental papilla and surrounding dental follicle. They also showed that the odontoblasts, dentine matrix and much of the pulpal tissue are of CNC origin. As development proceeds, the dental pulp becomes further populated with cells of non-neural crest origin, and such cells are most likely derived from the first branchial arch mesenchyme. Thus, the mature pulp comprises a heterogeneous population of cells (Figure 1-5).

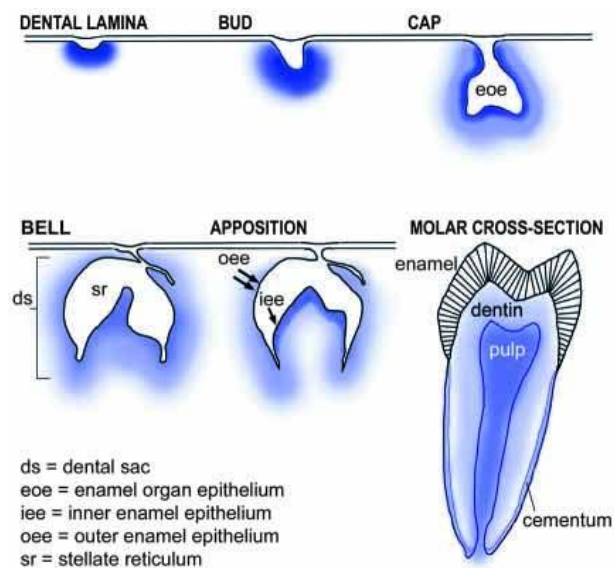


Figure 1-5: Schematic drawing of the life cycle of a tooth with contribution from CNC cells. When tooth development is initiated with the formation of the dental lamina, its underlying mesenchyme is almost entirely populated with CNC-derived cells (dark blue). As the tooth develops from the bud to cap stage, CNC-derived cells are concentrated (dark blue) at the interface with the epithelium, while the peripheral portion of the dental sac is less populated (light blue) with CNC-derived cells. In adulthood, CNC-derived cells contribute to the formation of dentine, pulp and cementum (Chai et al., 2002).

1.3.5.3 Stem cell niches in dental pulp

Several stem cell niches have been postulated within the dental pulp (Figure 1-6). Early studies have indicated that replacement odontoblasts, which synthesise reparative dentine in response to an injury, are derived from undifferentiated mesenchymal cells in the pulp proper. Labelling of pulp cells with tritiated thymidine following pulp capping showed an initial proliferation of cells in a deep pulpal area below the site of injury, followed by an apparent migration of these cells to the wound site where they underwent further proliferation and differentiation into functional odontoblast-like cells (Fitzgerald et al., 1990). Although the origin of those cells remained uncertain, Fitzgerald and co-workers proposed that these progenitor cells are derived from a population of fibroblast-like cells. They also noted a potential contribution from daughter cells derived from the perivascular cell population. Other studies monitoring *ex vivo* 5-bromo-2'-deoxyuridine (BrdU) uptake by proliferative cells in response to injury, have suggested that the progenitor/stem cell niches reside predominantly in the perivascular regions of the pulpal cavity, from where they migrate to the site of injury (Tecles et al., 2005, Feng et al., 2011). Expression of the stem cell markers CD146 and STRO-1 in the dental pulp is restricted to the blood vessel walls and is absent in surrounding fibrous tissues, odontoblast layer and perinurium of the nerves, suggesting that pulp stem cells are localized in the perivascular region of the pulp tissue (Shi and Gronthos, 2003). However, increased Notch expression following pulpal injury *in vivo* has revealed new niches in addition to the perivascular niche (Lovschall et al., 2005). Notch is thought to be an important signalling molecule that controls stem cell fate. In mammals, four extracellular Notch receptors (Notch 1–4) have been described. They interact with membrane-bound ligands that are encoded by the Delta (Dll1, Dll3 and Dll4) and Jagged (Jag1 and Jag2) gene families (Sullivan and Bicknell, 2003). One study in rats explored Notch expression following pulp capping. It was found that 1–3 days

after pulp capping, Notch 1 expression was increased in the odontoblast and sub-odontoblast layers; Notch 2 expression increased in the pulp stroma, whereas Notch 1 and 3 increased in cells associated with perivascular structures. These findings suggest that the progenitor/stem cell niches reside in different locations throughout the pulp tissue, and that the responsiveness of each of the progenitor/stem cell niches to injury varies according to its location (Lovschall et al., 2005).

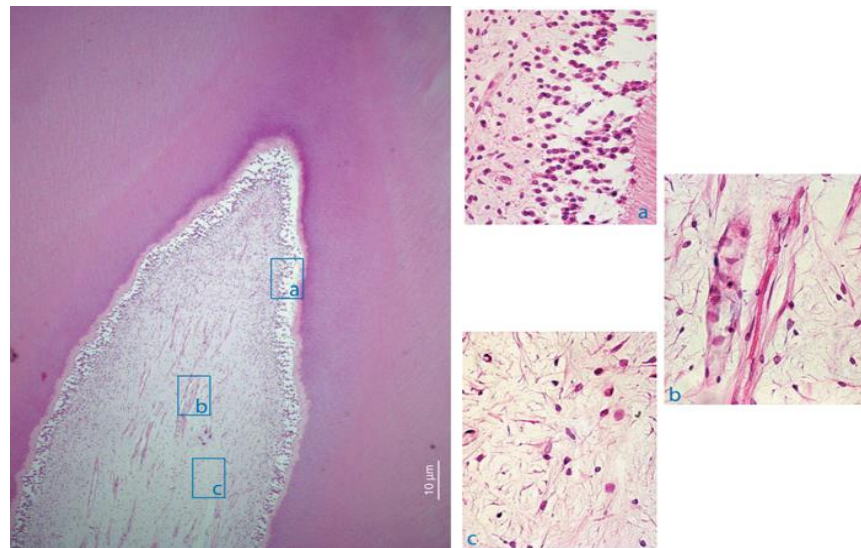


Figure 1-6: Stem/progenitor cell niches in dental pulp. (a) undifferentiated mesenchymal cells (so-called sub-odontoblasts) residing in the cell-rich layer close to the odontoblasts; (b) a perivascular cell population associated with the pulpal vasculature; and (c) a *Notch-2* positive cell population within the central pulpal stroma (Sloan and Waddington, 2009).

1.3.5.4 Dental pulp stem cells

There is, in the dental pulp of adult teeth, a population of clonogenic cells with a high proliferative capacity, the DPSCs. These cells were successfully isolated by enzymatic digestion of pulp tissue after separating the crown from the roots (Gronthos et al. 2000). Dental pulp stem cells are multipotent cells that proliferate extensively, can be safely cryopreserved, possess immunosuppressive properties, and express markers such as

CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146 and STRO-1, but do not express CD14, CD24, CD34, CD45, CD19 and HLA-DR (Huang et al., 2009, Lindroos et al., 2008). The plasticity of DPSCs has been verified through *in vitro* and *in vivo* studies. DPSCs have the ability to differentiate into odontoblast-like cells, osteoblasts, adipocytes, neural cells, cardiomyocytes, myocytes and chondrocytes *in vitro* (d'Aquino et al., 2007a, Zhang et al., 2006, Carinci et al., 2008, Arminan et al., 2009). Also, DPSCs can form mineralized nodules with a dentine-like structure under osteoinductive conditions *in vitro* and reparative dentine-like tissue on the surface of human dentine *in vivo*. DPSCs transplanted with HA/TCP produce a dentine-like structure lined with human odontoblast-like cells and surrounded by pulp-like interstitial tissue *in vivo* (Gronthos et al., 2000a).

1.4 Mouse incisor

1.4.1 Development and structure of the continuously growing mouse incisor

The mouse incisor exhibits special developmental features that allow for continuous growth at the apical end to compensate for attrition at the incisal edge. Early stages of incisor development are identical to those of molar teeth. However, during the cap stage (E14.5), the incisor rotates and pursues its development horizontally along the proximo-distal axis of the mandible. The labial epithelium, which will produce differentiated ameloblasts, grows longer in length than the lingual epithelium and the cervical loop forms at the end of the labial epithelium (E16.5, early bell stage). Subsequently, unlike the molar tooth germs, the incisor germs continue to grow permanently without forming a root (E19.5, late bell stage) (Harada et al., 2002) (Figure 1-7).

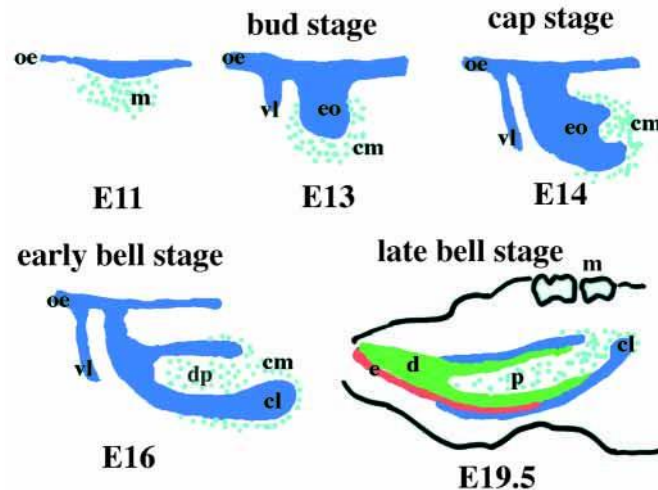


Figure 1-7: The developing mouse incisor during the initiation (E11), bud (E13), cap (E14), early bell (E16) and late bell (E19.5) stages. The initial stages of morphogenesis are very similar in all teeth. The first morphological sign of incisor development is a thickening of the oral epithelium (E11), which subsequently buds into the underlying mesenchyme (E13, bud stage). At later stages, the developing incisor rotates anteroposteriorly (E14, cap stage) and becomes parallel to the long axis of the incisors (E16, early bell stage). At early bell stage (E16), the cervical loop is seen at the apical end of the labial epithelium. Only the labial epithelium gives rise to the enamel forming ameloblasts (E19.5, late bell stage). Epithelium in dark blue, dental mesenchyme in light blue dots, enamel in red and dentin in green. cl, cervical loop; cm, condensed mesenchyme; d, dentine; dp, dental papilla; e, enamel; eo, enamel organ; m, molar; oe, oral epithelium; p, pulp; vl, vestibular lamina (Harada et al., 2002).

The lingual and labial epithelia that surround the mesenchymal pulp are distinct. The lingual side of the incisor is thinner and shorter than the labial side of the incisor. The labial side of the incisor is composed of four epithelial cell layers: the inner and outer enamel epithelia that surround the core of stellate reticulum and stratum intermedium. The inner and outer enamel epithelia are in contact with the mesenchyme of the pulp and the dental follicle respectively. The star-shaped cells of the stellate reticulum cells create a network, while the cells of the stratum intermedium are compressed, flat to cuboidal cells, which are in contact with the inner enamel epithelium. The labial cervical loop located at the apical end of the labial epithelium, consists of a core of stellate reticulum cells surrounded by the basal epithelium contacting the dental mesenchyme (Figure 1-8). Whereas the lingual cervical loop is very thin and contains only a few stellate reticulum cells. Enamel is deposited only on the labial side of the incisor, which is thus analogous to

the crown of a molar, while only dentine and cementum are deposited on the lingual side of the incisor, which provides a secure attachment of the tooth to the alveolar bone. This is why the lingual side of the rodent incisor is known as the root analogue of the molar. (Wang et al., 2007, Harada et al., 2002).

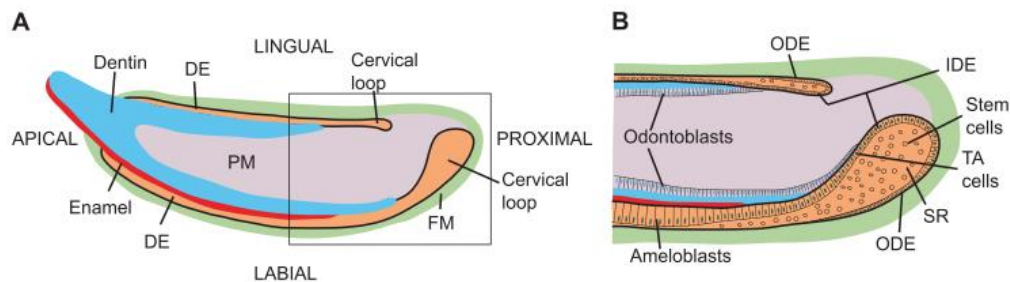


Figure 1-8: Schematics of the continuously growing mouse incisor.

(A) Basic overall organization of the incisor tooth. Growth occurs from proximal to apical (incisal) end. At the proximal end lie the lingual and labial cervical loops, each containing epithelial stem cells.

(B) Enlargement of boxed region in (A) showing the individual cell types that comprise the proximal (apical) end. Stem cells reside within the stellate reticulum, in the core of the labial cervical loop. See text for details. Color coding is as follows: enamel (red), dentin (blue), epithelium (orange), and follicular mesenchyme (Petersen et al.). DE, dental epithelium, FM, follicle mesenchyme, IDE, inner dental epithelium, ODE, outer dental epithelium, PM, papilla mesenchyme, SR, stellate reticulum, TA, transit amplifying (Wang et al., 2007).

1.4.2 Stem cells within the mouse incisor

The continuous growth and eruption of the rodent incisor suggested that stem cells are present in it. Cells in the apex of the incisor divide rapidly, compared with cells located closer to the incisal region and there is a gradient of cell differentiation in an apical-to-incisal direction (Smith and Warshawsky, 1977, Smith and Warshawsky, 1975). Hence, the incisor apex is thought to be the reservoir for the cells producing enamel and dentine (Hidemitsu Harada and Thesleff, 1999). Two types of stem cells exist in the mouse dental pulp, epithelial stem cells that give rise to enamel producing cells, ameloblasts and

mesenchymal stem cells that provide dentine forming cells, odontoblasts (Seidel et al., 2010a). Three stem cell niches were identified in the apical area of the mouse incisor: a cervical loop niche (Hidemitsu Harada and Thesleff, 1999) which is the source of epithelial stem cells, a perivascular niche (pericytes) and MSCs of a non-pericyte origin which is the resource of mesenchymal stem cells (Feng et al., 2011).

1.4.2.1 Stem cells in the cervical loop

Several lines of evidence indicate that the labial cervical loop contains the stem cell niche that houses the ameloblast progenitors. The first evidence comes from labelling experiments in *in vitro* cultures of mouse incisors. When cells in the centre of the cervical loop are labelled with the fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), the dye is first seen in the differentiating cells after 1 day and then in the ameloblasts after 2 days of culture, indicating that the ameloblast precursors reside in the cervical loop (Harada et al., 1999). This was supported by the analysis of BrdU incorporation during extended time periods, showing that the labelled cells extended progressively more incisally and occupied the zone of post-mitotic mature ameloblasts (Harada et al., 1999). Another *in vitro* analysis showed that when the labial cervical loop is removed mechanically from the apex of the tooth, the remaining epithelium could not regenerate the cervical loop. In these explants, all epithelial cells differentiated and produced mineralized matrix forming a stunt end with no indication of new cervical loop formation. Conversely, when the differentiated epithelium was removed instead of the cervical loop epithelium, the remaining cervical loop generated a new epithelium differentiating into secretory ameloblasts, thus indicating that it contains a pool of immature cells competent to regenerate the dental epithelium (Harada et al., 1999).

In the same study, slowly dividing putative stem cells were identified in organ culture experiments by labelling the cells of the incisor apex with BrdU for 3 hours, followed by a 7-day chase period. Labelled cells were detected in the cervical loop among the stellate reticulum cells, close to the basal epithelium. This location is consistent with the strong expression of Coxsackie Virus and Adenovirus Receptor (CVADR), a putative stem cell marker in brain cells (Johansson et al., 1999), in the labial cervical loop stellate reticulum (Harada et al., 1999).

The dental mesenchyme appears to be involved in the maintenance of epithelial stem cell populations within the cervical loop (Hidemitsu Harada and Thesleff, 1999). Fgf10, which is expressed in mouse incisor mesenchymal cells, is a survival factor that maintains epithelial stem cell populations in developing incisor germs, as in Fgf10 null mice, their incisors showed a small cervical loop when compared to the wild type at E16, while at later stages the cervical loop was missed (Harada et al., 2002). FGF9 was also involved in this process, therefore, the epithelial stem cell niche in growing incisors is maintained by an epithelial-mesenchymal interaction that involves Fgf9 and Fgf10 signalling (Tamaki Yokohama-Tamaki, 2008).

1.4.2.2 Perivascular and non-perivascular mesenchymal stem cells

Using Cre mediated genetic lineage tracing of pericytes, NG2cre mice were crossed with ROSA26r (R26R) reporter line. Pericytes were shown to differentiate into specialised odontoblasts during tooth growth and in response to damage *in vivo*. However, in both these situations, pericytes did not account for all of the cell differentiation as only 15% of the newly formed odontoblasts were pericyte derived. This suggested an additional source of MSC cells of a non-pericyte origin. (Feng et al., 2011). Using Dil labelling in different regions in the mouse incisor pulp to identify any responses to tooth damage, cells labelled

in the cervical area migrated to the damaged area after 2 days, while they remained quiescent in the absence of damage. This identified a distinct population of mesenchymal stem cells that are resident near the cervical opening of the incisor (Figure 1-9).

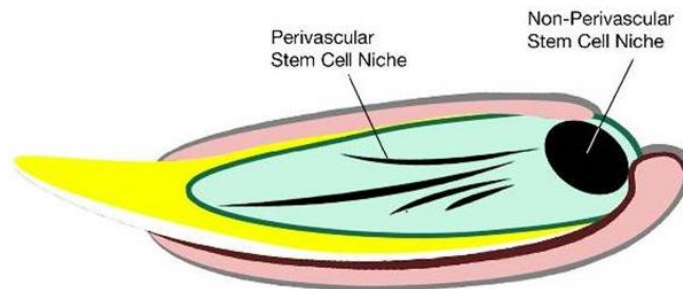


Figure 1-9: Two populations of mesenchymal stem cells reside in the dental pulp. The pericytes in a perivascular location and the mesenchymal stem cells at the apical end of the mesenchyme (Feng et al., 2011).

1.5 Mesenchymal stem cell markers

Although no unique markers are known for MSCs, their cell-surface antigen profile has been well explored (Kolf et al., 2007b). Research on MSC isolation has utilised monoclonal antibodies to pre-select cells with an MSC surface phenotype. The methods

investigated varied from negative selection, where other cell types, such as hematopoietic cells, are removed (Baddoo et al., 2003) to positive selection, where MSCs are directly enriched from a pool of other cells in which they are known to be present (Jones et al., 2002, Quirici et al., 2002, Jones et al., 2006). This proves very challenging in view of the lack of specific markers and the phenotypic plasticity that MSCs demonstrate *in vitro* (Jo et al., 2007).

The minimal criteria proposed by ISCT to define human MSCs (Horwitz et al., 2005) is that cells must be positive for CD105, CD73 and CD90 and negative for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR. These criteria allow only a retrospective definition of a cell population containing MSCs but do not allow prospective purification of MSCs. In addition, these criteria are not entirely valid across and between species. For example, in mouse models, MSCs frequently differ not only from the human MSCs, but also between strains in terms of marker expression and behaviour in culture (Peister et al., 2004, Sung et al., 2008). Indeed, most of these markers are expressed by cultures of fibroblastic cells from any tissue. In addition, most if not all of these markers are highly modulated in culture, which made efforts to characterize stromal cell cultures futile (Bianco et al., 2008). So, the identification of a definitive marker, allowing prospective isolation of MSCs from fresh tissue would be of the utmost importance. Current research has identified a variety of molecules that could be useful in the *in vivo* identification and purification of MSC-like cells (Jones and McGonagle, 2008). Among them are LNGFR (CD271), a neural marker repeatedly found to be expressed by MSCs (Jones et al., 2002, Quirici et al., 2002), and CD49a (Deschaseaux et al., 2003, Jones et al., 2006) together with the markers already indicated by the ISCT. Recently, PDGFRa⁺ Sca-1⁺ CD45- TER119- cells have been isolated from murine BM with abilities and characteristics consistent with conventional plastic-adherent MSCs (Morikawa et al., 2009). However,

no universally accepted assay has been established to date to probe for MSCs *in vivo* (Nombela-Arrieta et al., 2011a).

Thy-1 (CD90) is one of the best recognized mesenchymal stem cell markers. It has been used as a marker of stem cells in several tissues such as liver (Petersen et al., 1998), BM (Mayani and Lansdorp, 1994), and epidermis (Nakamura et al., 2006). It has also been suggested as a marker of neural and osteoprogenitor cells (Locatelli et al., 2003, Nakamura et al., 2010). In teeth, dental pulp stem cells express *Thy-1* and it was enriched in hard tissue forming progenitor cells (Balic et al., 2010, Balic and Mina, 2010). Also *Thy-1* expressing cells in human dental pulp stem cells were easily induced to mineralize (Karaöz et al., 2010, Pivoriūnas et al., 2010).

In mouse incisor dental pulp, mesenchymal stem cells had been located in the periapical mesenchyme and perivascular area (Feng, et al. 2011). Thy-1 is one of the pericyte markers (da Silva Meirelles, et al. 2008) which is considered a potential mesenchymal stem cell inside the dental pulp (Feng, et al. 2011). Yet Thy-1 expression has not been investigated in mouse incisor dental pulp. So Thy-1 was selected for further analysis.

1.6 Thy-1

Thymocyte differentiation antigen 1 (Thy-1), also known as Cluster of Differentiation 90 (CD90) is a Glycosyl Phosphatidyl Inositol (GPI) anchored cell surface protein that has been conserved throughout evolution, suggesting an important function for this molecule. *Thy-1* homologs have been described in many species, including squid, frogs, chickens, mice, rats, dogs, and humans (Williams and Gagnon, 1982, Pont, 1987).

The locus for murine Thy-1 maps to chromosome 9 and includes two alleles termed Thy-1a and Thy-1b, which code for Thy-1.1 and Thy-1.2 glycoproteins, respectively. Thy-1.1 and Thy-1.2 differ solely in amino acid position 89, which is occupied by Arginine in

Thy1.1 and by Glutamine in Thy-1.2 (Williams and Gagnon, 1982). Thy-1.2 is expressed by most mouse strains, whereas Thy-1.1 is an alloantigen of the AKR/J and PL strains. In humans, the gene coding for THY-1 is located in chromosome 11q22.3 (Seki et al., 1985b). Thy-1 is a somewhat enigmatic molecule that can exist in both membrane-bound and soluble forms.

1.6.1 Thy-1 protein structure

The core protein of 25-37 kDa Thy-1 consists of 111 or 112 amino acids. Rodent Thy-1 (Figure 1-10) is N-glycosylated at three sites (Almqvist and Carlsson, 1988a) while human Thy-1 contains only two glycosylation sites (Seki et al., 1985a). Thy-1 is one of the most heavily glycosylated membrane proteins, with a carbohydrate content up to 30% of its molecular mass (Almqvist and Carlsson, 1988a). The composition of Thy-1 carbohydrate moiety varies considerably between different tissues in the same animal, and even among cells of the same lineage at different stages of ontogeny. For instance, galactosamine is present only on brain Thy-1, whereas the sialic acid content of thymic Thy-1 far exceeds that of brain Thy-1 (Neil Barclay et al., 1976). Comparative analysis of thymocytes and lymph node cells have revealed that the sialic acid content of Thy-1 increases in parallel with T cell maturation (Hoessli et al., 1980).

Human and mouse Thy-1 are both initially translated as a 161 and 162 amino acids pro-form, respectively. The first 19 amino acids of Thy-1 acts as a signal peptide that targets it to the cell membrane and it is later removed. Then, Thy-1 is kept at the cell surface by 132-161 aa, which is embedded into the membrane. However this C-terminal transmembrane domain is cleaved away and a GPI moiety is added at residue 131 (Seki, et al. 1985a).

The GPI moiety is composed of two fatty-acyl groups that join Thy-1 to the cell surface and participate in targeting lipid rafts (Seki et al., 1985b). A soluble variety of Thy-1 exists and is presumably produced by a proteolytic (Park et al., 1999) and/or lipolytic cleavage at the cell surface. If the latter is the case, it is presumed to occur in close proximity to the GPI or within the GPI moiety itself. As for targeting within the GPI, both GPI-specific phospholipase D (GPI-PLD) and phospholipase C (GPI-PLC) can do so. The former is produced in mammals while the other is bacterial in origin. They have a distinct enzymatic requirement to cleave Thy-1 away from the cell surface. Exogenous GPI-PLD needs detergents to cleave Thy-1 while GPI-PLC does not (Bergman and Carlsson, 1994). However, susceptibility of Thy-1 to cleave differs from one cell type to another. Thy-1 positive endothelial cells and circulating T cells are susceptible to cleaving by serum GPI-PLD, but localization of Thy-1 to cholesterol rich lipid rafts is thought to protect it from GPI-PLD present in serum (Bergman and Carlsson, 1994).

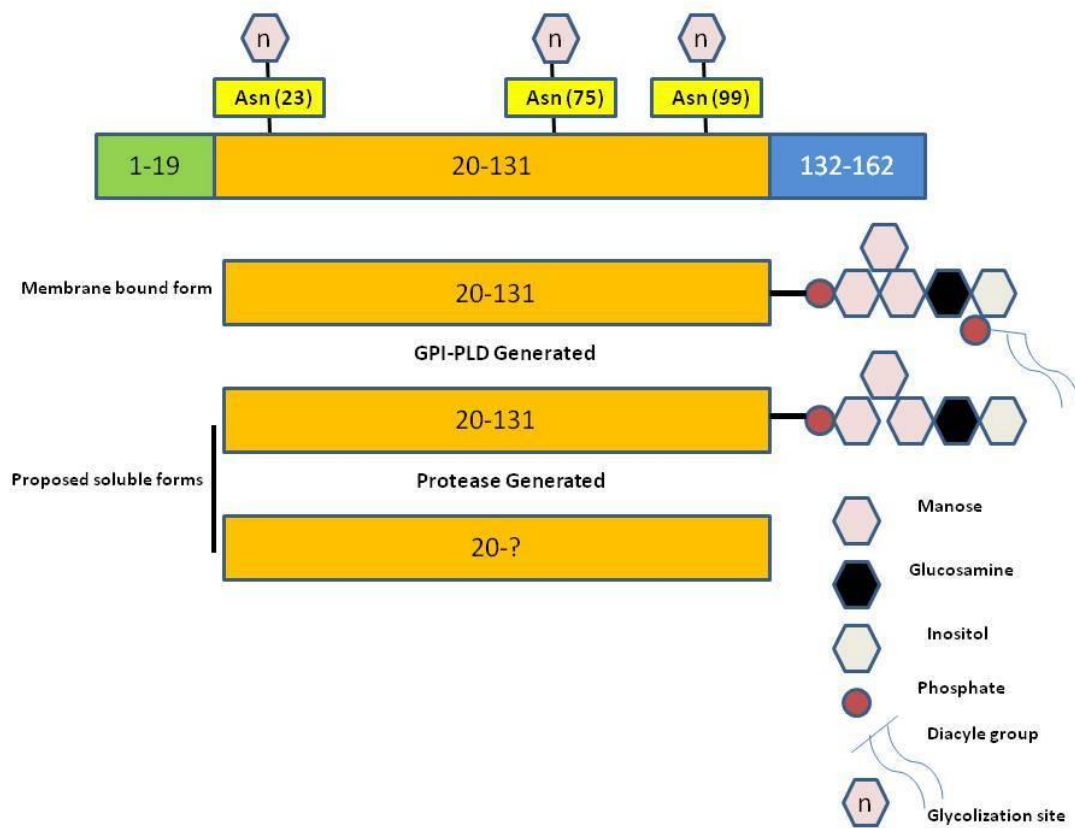


Figure 1-10: Murine Thy-1 molecule and proposed soluble forms. *Thy-1* is initially generated as a 161 aa pro form. The initial 19 aa signal peptide is removed, and the terminal 31 aa is replaced with a GPI anchor, generating the mature form, which is anchored to the outer leaflet of the cell membrane by the diacyl group of the GPI anchor. N-linked glycosylation sites depict conserved asparagines within murine *Thy-1* that are known to be glycosylated. Soluble *Thy-1* could be generated either by cleavage of the GPI anchor by GPI-PLD, or by undefined proteases acting at as yet undetermined cleavage sites (Bradley et al., 2009).

1.6.2 Thy-1 expression

Thy-1 is present on brain cells and fibroblasts of all species (Pont, 1987). In the mouse, Thy-1 is also found on a variety of other cell types including thymocytes, peripheral T cells, myoblasts, epidermal cells, and keratinocytes (Reif and Allen, 1964, Pont, 1987, Nakamura et al., 2006). Whereas in humans, Thy-1 is expressed by endothelial cells, smooth muscle cells, a subset of CD34 bone marrow cells, and umbilical cord blood- and fetal liver-derived hemopoietic cells (Mason et al., 1996).

Thy-1 expression in the nervous system is predominantly neuronal, but some glial cells are also Thy-1 positive especially at the later stages of their ontogeny (Kemshead et al., 1982). Thy-1 expression in the brain is developmentally regulated, as Thy-1 levels in the neonatal rat brain and developing human brain are low in comparison with the adult brain (Barclay, 1979). Thy-1 levels increase dramatically during the first few weeks of postnatal development in parallel with brain maturation (Seeger et al., 1982).

Lymphoid tissues of different species show a great deal of variation in Thy-1 expression. Thy-1 is probably the most abundant glycoprotein of murine thymocytes, with about 1,000,000 copies/cells covering up to 10–20% of the cell surface (Killeen, 1997). Cortical thymocytes express higher levels of Thy-1 than medullary thymocytes, whereas lymph node cells possess considerably less Thy-1 (200,000 copies/cell) in comparison with thymocytes (Pont, 1987). An inverse relationship between *levels of Thy-1 expression* and T cell differentiation is seen in rats, although rat Thy-1 is lost at an earlier stage of T cell maturation (Crawford and Barton, 1986). In humans, Thy-1 expression is restricted to only a small population of cortical thymocytes (McKenzie and Fabre, 1981).

1.6.3 Regulation of Thy-1 expression

The expression of Thy-1 is developmentally and spatially regulated. During early neuronal development in mice, Thy-1 expression is low but it increases with maturation (Barlow and Huntley, 2000). In rats and mice, Thy-1 protein is present on the cell body and dendrites of neurons but is not expressed on axons until axonal growth is complete (Xue et al., 1991). Also, during rat ovarian development, although Thy-1 is continuously expressed in the fallopian tube tunica propria, high levels of Thy-1 are expressed in the theca interna of growing antral follicles and developing corpora lutea. Moreover, Thy-1 is released by vascular pericytes in the ovaries during follicular differentiation (Bukovsky et

al., 1983). The same study, authors noticed that this decrease in Thy-1 occurred concurrently with the appearance of macrophages in the follicular antrum which raised the probability if the recruited macrophages were the source of proteases and cytokines that may stimulated Thy-1 shedding?

Expression of Thy-1 is also affected by injury. Two days post crush injury of the sciatic nerve, Thy-1 expression in dorsal root ganglia is decreased and returns between days 4 and 7 (Chen et al., 2005). Thy-1 mRNA expression, as assessed by RNase protection assays and *in situ* hybridization, is also down-regulated after optic nerve crush or intravitreal (inside eye) injection of N-methyl-D-aspartate (NMDA) to induce ganglion cell death (Schlamp et al., 2001).

Thy-1 expression differs by species, and this may be due to transcriptional regulation. In mice, Thy-1 is expressed on thymocytes and splenocytes, but is only expressed on thymocytes in rats. This might be due to the fact that the third intron of the mouse Thy-1 gene contains a 36 base pair region that can bind nuclear transcription factors, such as E Twenty Six 1 (Ets-1)-like nuclear Factor (NF), which is expressed in thymocytes and splenocytes. The homologous 36 base pair region of the rat Thy-1 gene does not contain the Ets-1 - like NF binding site, but binds another NF that is expressed only in rat thymocytes. Also, hypermethylation may lead to a decrease or absence of Thy-1 expression in Nasopharyngeal carcinoma (NPC) cell lines (Lung et al., 2005).

Thy-1 mRNA expression is rapidly followed by protein expression in rat cerebellum and rat and mouse cerebrum, whereas a delay of several days occurs between Thy-1 mRNA and protein expression in mouse Purkinje cells, indicating post-transcriptional regulation of Thy-1 expression (Xue and Morris, 1992).

Post-transcriptional regulation of Thy-1 may be due to a widely expressed transacting suppressor protein, as Thy-1.1 expression is down-regulated in heterokaryons (cell fusions) constructed with Thy-1.1-expressing mouse T cells and Thy-1.2-expressing mouse neurons (Saleh and Barlett, 1989). Therefore, the secreted suppressor of Thy-1 in neurons can also inhibit Thy-1 expression on T cells and is not tissue specific.

Iron levels also affect Thy-1 expression. Iron chelation in pheochromocytoma (rare tumor of adrenal gland tissue) cells propagated *in vitro* down-regulates Thy-1 expression. *In vivo*, rats fed an iron-deficient diet and brain tissue sections from patients with Restless Legs Syndrome, which is characterized by decreased iron levels in the substantia nigra (a brain structure located in the midbrain that plays an important role in reward, addiction, and movement), display decreased expression of Thy-1 (Wang et al., 2004).

Thy-1 expression may be regulated by protein shedding (Almqvist and Carlsson, 1988b). Elevated levels of soluble Thy-1 are detected in the serum and inflamed synovial fluid of patients with venous leg ulcers (Saalbach et al., 1999). Cytomegalovirus infection down-regulates Thy-1 expression on human dermal fibroblasts, through a mechanism that likely involves shedding and requires cytomegalovirus immediate-early/early gene products (Leis et al., 2004). Inflammatory cytokines such as Interukin (IL)-1 and Tumor Necrosis factor (TNF) induce shedding of Thy-1 from human lung fibroblasts associated with myofibroblastic differentiation (Hagood et al., 2005).

1.6.4 Thy-1 function

A large number of functions for Thy-1 have been described in a variety of interesting studies over decades. Thy-1 has been reported to function in T-cell activation, neurite

outgrowth, apoptosis, tumor suppression and wound healing-fibrosis (Haeryfar and Hoskin, 2004).

1.6.4.1 T- Cell activation

The abundance of Thy-1 expression, its signalling properties, and the stimulatory effect of Thy-1 cross-linking on mouse T-cells indicate that Thy-1 is more than just a T-cell marker (Haeryfar and Hoskin, 2004). T-cell receptor (TCR) signalling leads to T-cell activation and Interleukin-2 (IL-2) production in a complex process that centers on the immunological synapse (IS) in which Thy-1 acts as a co-stimulatory molecule to augment T-cell responses (de Mello Coelho et al., 2004) without cytotoxic effector function (Haeryfar et al., 2003). However, in the absence of TCR ligation and the presence of strong co-stimulation, Thy-1 is able to, at least partially, substitute for TCR-induced signalling (signal 1) during T-cell activation (Revy et al., 2001). This dual signalling capacity of Thy-1 depends on the availability of signalling via other cell surface molecules. Interestingly, it has been hypothesized that Thy-1-driven T-cell expansion, without the induction of a fully functional lytic phenotype, may constitute a mechanism for the maintenance of T-cell homeostasis without the risk of developing cell-mediated autoimmunity (Haeryfar and Hoskin, 2004). However, the signals that govern T-cell homeostasis under normal or lymphoid-poor states are not well understood (Figure 1-11).

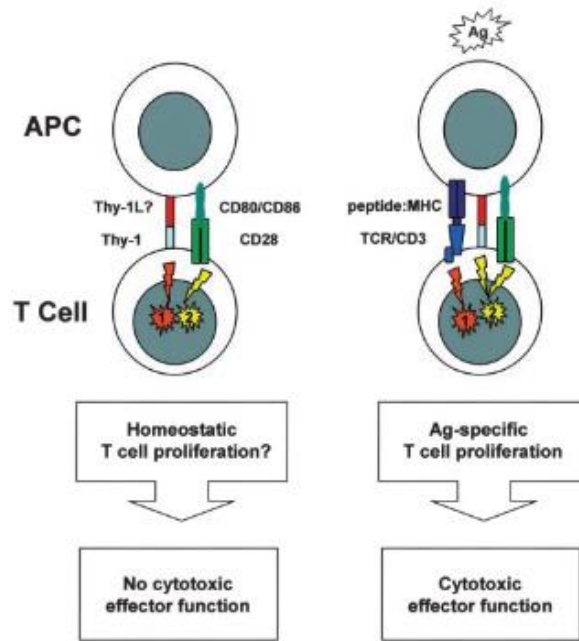


Figure 1-11: A hypothetical model for dual signalling by Thy-1. In the absence of TCR triggering by peptide:MHC complexes, Thy-1 engagement on mouse T-cells by a putative Thy-1 ligand (Thy-1L) on an APC (e.g., DC) provides a surrogate form of signal 1 to the T-cell. Concomitant provision of signal 2 through costimulatory CD28-CD80/CD86 interactions allows Thy-1-induced signal 1 to trigger T-cell proliferation, which may contribute to the maintenance of T-cell homeostasis. Thy-1 signaling does not trigger cytolytic effector function, thereby avoiding potentially harmful bystander cytotoxicity. In contrast, Thy-1 engagement in the context of TCR triggering by peptide:MHC acts in concert with CD28 to provide T-cells with an enhanced costimulatory signal 2 that leads to CTL development and Ag-specific cytotoxic effector function (Haeryfar and Hoskin, 2004).

1.6.4.2 Thy-1 promotes cell death in thymocytes and mesangial cells

Thy-1 can promote cell death both *in vitro* and *in vivo*. Treatment of malignant mouse T-lymphoma CS-21 cells *in vitro*, with an anti-Thy-1 Antibody (Ab), increases intracellular free calcium levels and induces apoptosis despite up-regulation of the antiapoptotic bcl-2 protein (Fujita et al., 1996). After treatment with the Thy-1 Ab, proteases are activated and the expression levels of antiapoptotic bcl-2 family members (bcl-2 and bcl-X1) are decreased. However, Thy-1 must aggregate in order to signal thymocyte apoptosis (Fujita et al., 1997).

In vivo injection of an anti-Thy-1 Ab into rats induces kidney mesangial (specialized cells around blood vessels in the kidneys) cell death and the development of

glomerulonephritis. After treatment of mesangial cells with anti-Thy-1 antibodies, inositol triphosphate and intracellular calcium levels are increased through a mechanism requiring protein tyrosine kinases (He et al., 1991). The cell death induced by Thy-1 is not necessarily apoptotic. A study using electron microscopy found that treatment of glomerular mesangial cells with an anti-Thy-1.1 Ab induces necrosis, not apoptosis. Although chromatin condensation is detected, nuclear membrane disruption, cellular swelling and organelle degradation are seen. This phenotype is consistent with necrosis. Furthermore, acute inflammation is detected as there is no phagocytosis of dead cells (Mosley et al., 2000).

1.6.4.3 Thy-1 promotes transendothelial cell migration of leukocytes and melanoma cells

Thy-1 is suggested to have a role in regulating cell adhesion necessary for melanoma invasion and metastasis. Thy-1 facilitates the adhesion and migration of melanoma cells across an endothelial cell monolayer, as it promotes the adhesion of melanoma cells to endothelium through interaction with $\alpha v \beta 3$ integrin on the former cells (Saalbach et al., 2005). Similarly, Thy-1 may play a role in leukocyte recruitment and extravasation during inflammation through the interaction of Thy-1 with $\alpha X \beta 2$ and $\alpha M \beta 2$ integrins on leukocytes (Leyton et al., 2001).

1.6.4.4 Thy-1 modulates the fibroblast phenotype relevant to wound healing and fibrosis

Normal pulmonary fibroblasts are heterogeneous and can be divided into subpopulations on the basis of size and shape, cytokine profiles, lipid content and cell surface protein expression (Penney et al., 1992, Fries et al., 1994). The most extensively characterized *in vitro* model of fibroblast heterogeneity is based on the surface expression of Thy-1

(Phipps et al., 1989). Thy-1 may play a role in wound repair and fibrosis as Thy-1 protein expression in human fibroblasts as those from granulation tissue is up-regulated on days 3–6 post-injury (Saalbach et al., 1996). In addition, Thy-1 expression affects fibroblast proliferation (Hagood et al., 2002). Fibroblasts lacking Thy-1 accumulate a higher level of IL-1 receptor antagonist (IL-1Ra) which inhibits induced T-cell clone proliferation in culture (Silvera and Phipps, 1995).

The original description of Thy-1 subpopulations in fibroblasts indicated significant differences in cellular morphology. Thy-1⁻ murine pulmonary fibroblasts have a polygonal morphology and a well established microfilament and microtubule cytoskeleton compared to the more spindle-shaped Thy-1⁺ fibroblasts. These differences have been found to be linked to Thy-1-associated signalling, as Thy-1 expression has been reported to affect cell-matrix adhesions, stress fiber formation and migration (Phipps et al., 1989). Pulmonary fibroblasts sorted on the basis of Thy-1 expression differ in expression of and response to cytokines and growth factors, proliferation and cellular morphology. These differences suggest that Thy-1 expression may affect the function of fibroblasts in wound healing and fibrosis (Hagood et al., 2005).

1.6.4.5 Thy-1 is a tumor suppressor in ovarian cancer and nasopharyngeal carcinoma

In human ovarian cancer cell line SKOV-3, the transfer of chromosome 11 suppresses tumour growth in immunodeficient mice. *Thy-1* is expressed only in non-carcinogenic cell clones. Thrombospondin-1 (TSP-1), a secreted protein acidic rich in cysteine (SPARC) and fibronectin are also up-regulated in non carcinogenic clones while they are not expressed in SKOV-3 cells, and their expression levels are most likely regulated by gene(s) on chromosome 11 (Abeyasinghe et al., 2003). When fibronectin expression is lost

in tumours and angiogenesis is required for tumour growth and metastasis (Akiyama et al., 1995, Abeysinghe et al., 2003), *Thy-1* could potentially inhibit tumour growth by up-regulating the expression levels of fibronectin and TSP-1.

The role of *Thy-1* in nasopharyngeal carcinoma (NPC) was investigated. *Thy-1* expression is decreased or lost in multiple NPC cell lines, 65% of NPC samples, and 63% of lymph node metastatic NPC tumours. Loss of *Thy-1* expression appears to be due to promoter hypermethylation in NPC cell lines. Restoration of *Thy-1* expression in NPC cells decreases colony formation, which suggests the tumour suppressor function of *Thy-1* in NPC (Lung et al., 2005).

1.6.4.6 *Thy-1* modulates neurite outgrowth

Neurite outgrowth is important for neuronal growth and synapse formation. As axons cannot repair, understanding the molecular signaling in neurite outgrowth is important for the treatment of brain and spinal cord injuries, as well as neurodegenerative diseases. Expression of *Thy-1* in a neural cell line inhibits neurite outgrowth over a substratum of mature astrocytes, characteristic star-shaped glial cells, but not over Schwann cells or embryonic glial cells (Tiveron et al., 1992). These data suggest that *Thy-1* may function to stabilize neuronal synapses and block neuronal repair in astrocyte-rich regions of the brain.

Thy-1 engagement of the $\beta 3$ integrin on astrocytes induces focal adhesion kinase (focal adhesion kinase F-actin) (FAK), p130Cas, and RhoA activation and the recruitment of paxillin, vinculin, and FAK to focal contacts, promoting focal adhesion and stress fiber formation (Leyton et al., 2001) (Figure 1-12). The interaction of neuronal *Thy-1* with $\beta 3$ on astrocytes may promote bi-directional signalling, inhibiting neurite outgrowth (Avalos et al., 2002). However, there is also evidence that *Thy-1* activation promotes neurite

outgrowth as the addition of an anti-Thy-1 Ab can promote neurite outgrowth. This promotion of neurite outgrowth by Thy-1 is dependent on G α_i and L- and N-type calcium channel activation (Doherty et al., 1993). An unidentified Thy-1 binding protein on astrocytes is necessary for Thy-1-induced neurite outgrowth (Dreyer et al., 1995).

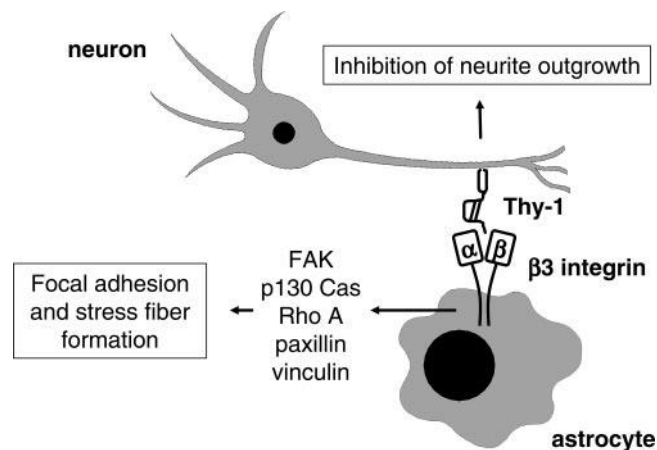


Figure 1-12: Bidirectional signalling activated by the interaction between Thy-1 on neurons and integrin $\beta 3$ on astrocytes. After an interaction between integrin $\beta 3$ and Thy-1, FAK, p130Cas, vinculin, paxillin, and RhoA are activated, promoting focal adhesion and stress fiber formation in astrocytes. This interaction may also be necessary for the inhibition of neurite outgrowth over astrocytes (Rege and Hagood, 2006).

1.6.5 Thy-1 mutant mice

Some of the functions of Thy-1 *in vivo* have been assessed using the Thy-1 knockout mouse. Thy-1 null mice are viable and appear neurologically normal, but display excessive GABAergic (Gamma-AminoButyric Acid) inhibition in the dentate gyrus (part of hippocampus contribute to new memories formation and known for its high rate of neurogenesis) and regional inhibition of long-term potentiation (LTP) which accounts for different types of learning (Nosten-Bertrand et al., 1996, Hollrigel et al., 1998). Additionally, these mice did not base their food choices on learned socially transmitted cues. Although Thy-1 null mice displayed normal social interactions and normal learning

in a maze, they failed to learn from other mice which foods were safe to eat when compared to wild type mice (Mayeux-Portas et al., 2000). However, failure to learn from social cues is rescued after transgenic expression of Thy-1 or pharmacologic treatment with a GABA (A) receptor antagonist. These data suggest that Thy-1 may regulate GABAergic inhibition of neurotransmission, which may affect the ability to learn from socially transmitted cues. Thy-1 null mice also had impaired cutaneous immune responses and abnormal retinal development when compared to the wild type control mice (Beissert et al., 1998, Simon et al., 1999). In the lung, loss of fibroblast Thy-1 expression correlated with lung fibrogenesis (Hagood et al., 2005) and recently, loss of Thy-1 inhibited alveolar development in the newborn mouse lung (Nicola et al., 2009). Phenotypic characterizations of craniofacial structures in Thy-1 null mouse are still lacking.

1.6.6 Thy-1 as a stem cell marker

MSCs are among the many cells that express Thy-1 (Bradley et al., 2009). Thy-1 is a stem cell marker known to be expressed by human bone marrow and cord blood CD 34⁺ hematopoietic progenitor cells (Mayani and Lansdorp, 1994). Furthermore, Thy-1 is widely used as a mouse hematopoietic stem cell marker (Petersen et al., 1998). In rats' livers, Thy-1 has been reported to be expressed by oval cells proliferating during stem cell-mediated regeneration. Consequently, Thy-1 has become an accepted cell surface marker to sort hepatic oval cells that are believed to be biopotential, i.e. able to differentiate into hepatocytes or bile ductular cells (Petersen et al., 1998). However, another study showed that Thy-1 protein was consistently present outside the basement membrane surrounding the oval cells and its expression overlapped frequently with smooth muscle actin-stained subpopulations of myofibroblasts/stellate cells (Dezso et al., 2007). Parallel with that, Thy-1 has been demonstrated as an *in vivo* and *in vitro* marker for the liver myofibroblasts (Dudas et al., 2007).

Regarding the neural lineage, Thy-1⁺ Sca-1⁺ mouse bone marrow derived-cells resulted in high Nestin positive spheres, when cultured in a neuronal differentiating medium. These spheres expressed mature neuronal markers like Tubulin III (TuJ-1) and Neurofilament (NF) and showed polarized morphology *in vitro* and *in vivo* (Locatelli et al., 2003). In addition, human muscle and rat BM- derived neurospheres are Thy-1⁺ (Kabos et al., 2002, Romero-Ramos et al., 2002).

Thy-1 has also been suggested as a good marker to characterize osteoprogenitor cells (Nakamura et al., 2010). Moreover, Thy-1 expression under cytokine stimulation could be an indicator of cell cycle phase in targeting Hematopoietic Stem Cells (HSC) as 40% of CD34^{high} CD38⁻ flt-3⁻ Thy-1⁺ cells entered S/G2M phase after 6 days of culture, independent of the Thy-1 expression of pre-cultured cells. On the other hand, CD34^{high} CD38⁻ flt-3⁻ Thy-1⁻ cells remained essentially quiescent, again independent of pre-culture Thy-1 expression (Takeda et al., 2005).

Thy-1 was indicated as a marker for the detection of human keratinocytes stem / progenitor cells enriched populations in cultured keratinocytes (Nakamura et al., 2006). By using *in vivo* human epidermal cyst formation assay, the injection of Enhanced Green Fluorescent Protein (EGFP)-labelled Thy-1⁺ or Thy-1⁻ keratinocytes in non-obese, diabetic/severe, combined immunodeficient (NOD/SCID) mice showed that EGFP⁺ cell cluster areas in the basal layer derived from EGFP⁺ Thy-1⁺ cells were eightfold larger than clusters of EGFP⁺ Thy-1⁻ cells. Furthermore, immunohistochemical staining indicated that Thy-1⁺ cells were expressed in most of the basal layer of the normal human epidermis.

Uncertainty arose about whether Thy-1 can absolutely segregate HSCs from multipotent progenitors (MPPs) (Notta et al., 2011). Although Thy-1⁻ and Thy-1⁺ populations

showed comparable self-renewal potentiality and long-term repopulating activity, Thy-1⁻ compartment was heterogeneous and contained a small fraction with repopulating activity and a larger fraction with MMPs-like activity. Consequently another surface marker i.e. CD49f combined with Thy-1 was used to purify HSCs. Recently, *Thy-1* was identified as a marker for cancer stem cells in primary high-grade gliomas using tissue microarrays (He et al., 2011).

1.7 Hypothesis and Aim of the research project

DPSCs constitute heterogeneous cell populations inside the dental pulp. The precise identity of DPSCs remains a challenge because of the lack of a single specific stem cell

marker. Thy-1 is shown to be expressed in some stem/progenitor cells in several tissues including teeth.

Based on the previous statements, we hypothesized that Thy-1 expressing cells in the mouse incisor dental pulp are stem cells. So, the aim of this project is to identify Thy-1 expression in mouse incisor dental pulp cells.

In the first chapter, Thy-1 expression in mouse incisor dental pulp will be investigated as follow:

- Detection of Thy-1 mRNA by whole mount in situ hybridization
- Detection of Thy-1 protein by immunohistochemistry.
- Detection of Thy-1 expression level in different cell passages by flow cytometry.
- Sorting of Thy-1 expressing cells using MACS and grow them in culture.
- Characterization of Thy-1 expressing cells using other mesenchymal stem cell markers
- *In vitro* osteogenic differentiation of mouse dental pulp parallel with Thy-1 expression level detection by flow cytometry.

In the second chapter, the contribution of Thy-1 expressing cells in mouse incisor will be investigated using lineage tracing in the following stages:

- Embryonic day 14.5 (E14.5)
- Embryonic day 17.5 (E17.5)
- Postnatal day 5(P5)
- Postnatal day 60 (P60)(Adult stage)

In the third chapter, Regulation of Thy-1 expression by Shh signalling pathway will be investigated as follow:

- *In vitro* using cell and organ culture
- *In vivo* using transgenic mice

Chapter 2 Materials and Methods

2.1 Reagents

Complete list of all reagents used in this study is found in the Appendix.

2.2 Molecular biology techniques

Plasmids, restriction enzymes and RNA polymerases used in this study are listed in table (2-1)

Table 2-1: Details of plasmids, restriction enzymes and RNA polymerases used to make anti-sense probe

Gene	Vector	Size of insert	Digestion Enzyme to Linearise Plasmid DNA	Polymerase Enzyme to Generate Antisense Probe
Crabp1	PT7T3D-Pac1	1.3Kb	Sfi1	T3
Hus-1	pSport1	2kb	Kpn1	Sp6
Thy-1	pCMV-SPORT6	2.3kb	Kpn1	T7
Ikros1	PT7T3D-Pac1	1.5kb	Pac1	T7
Shh	pBluescript	2.6 kb	EcoR1	T7
Gli-1	Bluescript	1.7 kb	Not1	T3
Ptc-1	pBluescript	1 kb	BamH1	T3

2.2.1 Transformation of competent E.coli cells with plasmid DNA

Approximately 1.0 µg of plasmid DNA was added to 50 µl of E.coli DH5α competent cells (Invitrogen,UK). The mixture was gently mixed and the cells were stored on ice for 30 minutes. The bacteria were then heat shocked by incubation for 60 seconds in a water bath at 42°C, followed by 2 minutes on ice. 450 µl of Luria-Bertani-medium (LB-medium) was added and the cells were incubated in a shaker at 37°C for 30 minutes. 50-100 µl of the mixture was streaked on a LB-agar plate containing 100 µg/ml Ampicillin (Sigma-Aldrich). The plates were then incubated overnight at 37°C. A single clone was used to inoculate LB-medium for maxiprep plasmid isolation.

2.2.2 Minipreparation of plasmid DNA

To isolate DNA for sequencing, plasmid DNA was isolated from Spin columns (Fast Plasmid® Mini) by following the protocols provided by the manufacturer (Eppendorf AG).

2.2.3 Maxipreparation of plasmid DNA

To isolate DNA for anti-sense probe, plasmid DNA was isolated using DNA purification columns from a Plasmid Maxi kit as recommended by the manufacturer (QIAGEN).

2.2.4 DNA quantification

Plasmid DNA concentration was determined by measuring absorbance of a 1/500 dilution of the DNA samples at 260 nm using a spectrophotometer (BioPhotometer, Eppendorf AG).

2.2.5 Preparation of template DNA to make antisense riboprobes

2.2.5.1 Linearization of plasmid DNA

20 µg of plasmid DNA were digested using appropriate restriction enzymes (Promega) in the reaction mixture listed below (Table 2-2). For making antisense probes, a unique restriction site located 5' to the sequence to transcribe was used. The reaction was incubated at 37°C for 3 hours. 0.2 µl of linearized DNA product (corresponding to 400 ng of linearized DNA) and an equivalent quantity of unlinearized DNA were then run on a gel to confirm the completion of the digestion.

Table 2-2: Reagents used for linearization of plasmid DNA (per reaction)

Reagents	Volume
Bovine serum albumin (10µg/µl)	0.5 µl
Plasmid DNA	20 µg
Restriction enzyme	2 U/ µg plasmid DNA
10x Buffer	5 µl
Nuclease-free H ₂ O	up to final volume (50 µl)

2.2.5.2 Purification of linearized plasmid DNA

Once completion of the digestion was confirmed by running of 0.5µl of digested DNA sample on agarose gel, the linearized plasmid DNA was purified by running the whole digested DNA on an agarose gel followed by purification using the QIAquick Gel Extraction Kit (QIAGEN) following manufacturer instructions.

2.2.6 Synthesis of DIG-labelled RNA Probes (DIG-RNA) by *In vitro* Transcription

Antisense RNA probe was synthesised from each linearised plasmid by adding reagents as outlined in (Table 2-3). The reagents were mixed well and incubated at 37°C for 2 hours and 1µl of the specific polymerase was added after the first hour. 1µl of the transcribed DNA was run on a gel to confirm the production of RNA after the reaction. 2 µl of RNase free DNase (Promega) was then added and the reaction was incubated at 37°C for 15 minutes to eliminate the DNA template. Following the DNase treatment, the synthesised RNA was purified with a SigmaSpin™Post-Reaction Clean-Up Column (Sigma-Adrich) following manufacturer instructions. The RNA probe was collected and stored at -80°C for further use.

Table 2-3: Reagents to Transcribe a DIG-labelled RNA Probe (per reaction)

Reagents	Volume
5x transcription buffer (Promega)	8 µl
100 mM DTT (MP Biomedicals)	4 µl
RNasin (40 U/µl) (promega)	1 µl
Linearised plasmid DNA	1 µg
DIG RNA labelling mix (Roche)	2 µl
Polymerase enzyme (20U/µl)	1 µl
Nuclease-free H ₂ O	up to final volume (40 µl)

2.3 *In situ* Hybridisation

2.3.1 Whole Mount DIG *In situ*

2.3.1.1 DIG Whole mount *In situ* Hybridisation

Postnatal teeth were carefully dissected out in cold Rnase free 1X PBS (Sigma-Aldrich) and were then fixed in 4% PFA (Sigma-Aldrich) at 4°C for overnight or up to 24 hours. The fixed teeth were then decalcified in 4% EDTA (VWR) containing 1% PFA for a few days at room temperature on a shaker. After decalcification, the tooth samples were thoroughly washed with ice-cold PBT (0.1% Triton X-100 in 1X DEPC PBS) for 5 minutes three times to eliminate residual PFA. The samples were then dehydrated in a graded methanol series diluted in PBT (25%, 50%, 75%, 100% and 100%), for 10 minutes in each solution. The samples were then stored at -20°C until use.

2.3.1.2 Pre-treatment and Hybridisation of Whole Tooth Sample

Whole tooth samples were then rehydrated in a 75%, 50% and 25% methanol/PBT series and washed in PBT for 5 minutes three times at room temperature. The developing teeth were treated with detergent mix for 3x20 minutes to permeabilize the tissues. The samples were then refixed in 4% PFA for 20 minutes and washed for 5 minutes three times in PBT at room temperature. Whole teeth were then briefly rinsed in a 1:1 mixture of hybridisation solution (Table 2-4) and PBT and then immersed in the pre-warmed hybridisation solution at room temperature until they sank. The teeth were then incubated with pre-warmed hybridisation solution at 70°C for at least 1 hour. For hybridisation, the developing teeth were incubated with pre-warmed hybridisation solution containing 0.1 µg of DIG-labelled RNA probe per ml of hybridisation solution with gentle rocking at 70°C overnight.

Table 2-4: Reagents Used for Hybridisation Solution (Whole Mount In situ Hybridisation)

Components	Volume
Formamide (Merk Millipore)	5 ml
20X SSC (3M NaCl 0.3M sodium citrate, pH4.5)	2.5 ml
Blocking Reagent (10%)(Roche)	2ml
10 mg/ml tRNA (Roche)	1ml
0.5 M EDTA, pH8	100 µl
50µg/µl Heparin (Sigma-Aldrich)	10 µl
10 % Triton X-100,BDH (VWR)	10 µl

2.3.1.3 Post-hybridisation Washes and Detection of the Probe

After hybridisation, unbound probe was removed by thorough washes with pre-warmed washing solution (50% formamide), 1X SSC pH 4.5 and 0.1% Tween 20 (Sigma-Aldrich) for 30 minutes four times at 65°C on a rocking plate. The teeth were then briefly rinsed in a 1:1 mixture of washing solution and MABT (100 mM Maleic acid pH 7.5, 150 mM NaCl and 0.1% Tween 20) at 65°C, followed by three washes in MABT for 5 minutes each and then another two 30 minutes washes at room temperature. The teeth were then blocked with 2% blocking reagent in MAB (100 mM maleic acid, pH 7.5, and 150 mM NaCl) for 1 hour at room temperature and then with 2% blocking reagent and 20% sheep serum in MAB for another 2 hours at room temperature. The probe was detected by incubating the tissue samples overnight at 4°C with a 1/2000 dilution of alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche) diluted in MAB with 2% blocking powder and 20% sheep serum. The following day, the teeth were washed six times in MABT for 1 hour each at room temperature and washed overnight at 4°C in MABT on a shaker.

Prior to color development, the teeth germs were immersed in freshly made NTMT solution (100 mM Tris-HCl pH 9.5, 50 mM MgCl₂, 100 mM NaCl and 0.1% Triton X-100) for 10 minutes four times at room temperature. The samples were then incubated in the dark with 1 µl/ml 4-Nitro blue tetrazolium chloride (NBT) (Roche) and 1 µl/ml 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche) in NTMT at room temperature with gentle shaking. Colour precipitates (bluish-purple) were produced at the sites of the target RNA by chemical reaction of NBT and BCIP substrates with alkaline phosphatase. The progress of the reaction was monitored periodically and the reaction was stopped by washing with NTMT and PBT for 10 minutes each. The whole teeth were then placed in PBT overnight at 4°C, photographed, and stored in 4% PFA at 4°C.

2.3.1.4 Vibratome sectioning

Fixed samples were washed briefly in 1X PBS and then embedded in the desired plane in prewarmed 20% gelatine (Sigma-Aldrich) by using plastic disposable moulds. The gelatin was then allowed to solidify overnight at 4 °C. Gelatin blocks were then fixed in 4% PFA for a week at 4 °C. For sectioning, gelatin blocks containing the sample were glued onto a metal block holder with super glue, allowed to dry, and then sectioned with a vibratome (Leica VT 1000S) while submerged in a 1X PBS bath. Individual sections (50 µm thickness) were collected with a fine brush and transferred to slides. The slides were mounted with coverslips using an aqueous mounting reagent. The mounted slides were cleaned to remove excess mounting agent before taking photographs using a Zeiss Axioscop microscope (Germany).

2.3.2 DIG-section *In situ* Hybridisation

2.3.2.1 Pre-treatment and Hybridisation of Tissue on Sections

RNase-free glassware, slide racks and metal spatulas were prepared by baking overnight in an oven at 180°C. All the solutions used were DEPC (Sigma-Aldrich)-treated and autoclaved. The slides bearing paraffin sections (Described in details in 2-11) were firstly de-paraffinized twice with histoclear for 10-15 minutes each. The sections were later rehydrated through the following ethanol solutions: 100% (2 minutes twice), 95% (2 minutes twice), 70% (2 minutes twice) and 1X PBS (10 minutes twice). After rehydration, the sections were incubated in 10µg/ml Proteinase K (Sigma-Aldrich) in 1X PBS at 37°C for 10 minutes to permeabilise the tissues. The sections were then incubated in 2 mg/ml glycine (Sigma-Aldrich) in 1X PBS at room temperature for 10 minutes to block the protease, rinsed with 1X PBS for 5 minutes followed by a re-fixation in 4% PFA for 20 minutes at room temperature. To remove the remaining positive charges in the tissue, sections were acetylated for 10 minutes in a solution made by adding 25µl acetic anhydride to 10ml 0.1 M Triethanolamine (VWR BDH Prolabo) immediately before use. The sections were lastly washed with 1X PBS for 5 minutes three times before hybridisation.

To undergo hybridisation, the sections were incubated in pre-warmed hybridisation solution (Table 2-5) at room temperature for 1 hour. The sections were placed horizontally on glass rods in chambers with tissue towels soaked in 50% formamide and 5X SSC. Approximately 20-50ng of DIG-labelled RNA probe diluted in 1 ml of hybridisation solution was denatured by heating at 100 °C for 3 minutes immediately followed by 3 minutes on ice before applying it to the slides. 100- 200 µl of probe diluted in hybridisation solution was applied to each slide and glass cover slips were placed on

the slides to evenly spread the probe. The chambers were sealed with cling film to maintain humidity in the box and incubated at 70°C overnight in a hybridisation oven.

Table 2-5 Reagents used for hybridisation solution (DIG-section *In situ* Hybridisation)

Components	Volume
Formamide	5 ml
1M Tris-HCl pH 7.6	0.5 ml
10 mg/ml tRNA	1ml
50X Denhardt's solution	1ml
50% Dextran sulphate(Chemicon Millipore)	5ml
5M NaCl	6ml
10% SDS (Sigma-Aldrich)	1.25
0.5 M EDTA, pH8	100 µl
Nuclease-free H ₂ O	Up to 50 ml

2.3.2.2 Post-hybridisation Washes and Signal Detection

Following hybridisation, the cover slips were removed by dipping in pre-warmed 5 X SSC solutions. To remove the unbound probe, the sections were washed in 2X SSC solution with gentle rocking at 70°C for 1 hour and then equilibrated in TBS buffer (100mM Tris-HCl and 150mM NaCl) at room temperature for 5 minutes. Subsequently, the sections were blocked with 0.01% blocking reagent in TBS for 1 hour at room temperature and then incubated in a 1: 5000 dilution of an anti-DIG antibody coupled with alkaline phosphatase in TBS blocking buffer overnight at 4°C to detect the probe. The following day, the sections were washed in TBS 5 minutes three times and then incubated in freshly made NTMT solution for 5 minutes at room temperature. Color signal (bluish purple) was developed in the dark at room temperature by incubating the sections with 2.5 µl/ml NBT and 1.7 µl/ml BCIP in a base solution (50% Poly(vinylalcohol) (72000,Sigma-Aldrich), 100mM Tris-HCl pH 9.5, 100mM NaCl,

5mM MgCl₂ and 0.1% Tween20). When the production of a strong purple colour was achieved, the reaction was stopped by rinsing with NTMT solution and immersing in 1X PBS for a while. The slides were then air-dried and mounted with cover slips using a DePex (VWR BDH Prolabo) mounting medium and photographed afterwards.

2.4 Optical Projection Tomography (OPT)

2.4.1 Embedding

After *in situ* hybridisation, the specimen was washed into phosphate buffered saline (PBS) and embedded in a supporting block of 1% low-melting point agarose (Sigma-Aldrich). This agarose block afterward was stuck on a magnetic mount with a cyanoacrylate adhesive (superglue). The block was trimmed with a sharp blade to give a smooth surface with many facets, leaving a few millimetres of agarose around the specimen.

2.4.2 Clearing

The specimen in its agarose block was dehydrated in 4 changes of 100% methanol (HPLC, Fisher) each for half a day. Due to dehydration, sample turned opaque, and was subsequently placed in a histological ‘clearing agent’ for few hours to make it transparent which optically matches the refractive index of cell membranes. Normally, this was a mixture of 2 parts of benzyl benzoate to 1 part benzyl alcohol (BABB) (Sigma-Aldrich), which has a refractive index 1.51.

2.4.3 Scanning

Before scanning a specimen, the scanning microscope needed to go through some alignment and calibration checks using the alignment pin. The specimen stuck to the mount (Bioptonic) was inverted and attached by magnetism to the rotating plate above the cuvette in the OPT microscope (Bioptonic). The door to the specimen chamber was closed

and the lowering of the specimen into the cuvette was controlled using the Skyscan scanner software.

2.5 Cell culture

2.5.1 Sample collection

With sterilized tweezers, 60 mandibular incisors were dissected out from the mandibles by gently removing the mucosa and the bone shell covering them. The dental pulp was then squeezed out of the tooth by pressing it gently with a curved needle in sterilized PBS (Sigma-Aldrich).

2.5.2 Explant culture

The dental pulp tissue was cut into small piece using a sharp sterilized blade. These pieces were transferred to a 6 well culture plate or T-75 tissue culture flask (BD Falcon) with α MEM (w/ UGln1 and nucleosides, Lonza Cologne GmbH) containing 20% serum (Lonza, DE14-801F), 2% L glutamine (Sigma-Aldrich) and 1% Pen/Strep (Sigma-Aldrich). Culture plates were kept in humidified chamber at 37°C. This passage was defined as passage 0 and later passages were named accordingly. This culture medium was then changed twice a week.

2.5.3 Subculture

The cells were passaged and trypsinized by Trypsin/EDTA (Sigma-Aldrich) once the cultured cells reach 70% confluency. All media was removed and cell monolayer was washed with sterilized PBS x 3. 1-5 ml Trypsin was added for 5-8 minutes at 37°C. When cells are detached, an equal amount of media containing serum was added to stop the effect of Trypsin. Cell suspensions were then centrifuged at 1200 relative centrifugal force (rcf) for 5 min at 4° c to get the cell pellet. Then the cell pellet was suspended in adequate amount of media and cells were re-plated a split ratio 1:2.

2.6 Flow Cytometry

2.6.1 Antibodies used in Flow cytometry are listed in table (2-6)

Table 2-6: Antibodies and their optimal dilution used in flow cytometry

Gene	Dilution	Gene Localization	Manufacture
Mouse monoclonal to Thy-1 CD 90 conjugated to Fluorescein Isothiocyanate (FITC)	1:100	Cell Surface	Abcam 62009
Mouse monoclonal [P1H12] to CD146 conjugated to Phycoerythrin (PE)	1:70	Cell surface	Abcam 75769
Rat anti mouse monoclonal to CD133 (Prominin-1) conjugated with Allophycocyanin (APC)	1:700	Cell surface	e- bioscience
Purified rabbit polyclonal to Patched / PTC	1:50	Cell surface	Abcam 53715
Mouse IgG2bk isotype control FITC	1:100	Cell surface	e-bioscience

2.6.2 Sample collection

For fresh tissue, dental pulps first were cut into small pieces and digested using Triple E (Gibco) for one hour at 37°c. The completion of digestion is checked under the optical microscope. Once completed, the reaction is stopped by adding FBS. Cell suspension then centrifuged at 1200 rcf for 5 minutes at 4° c to get the cell pellet which then resuspended in media and goes for plating or staining.

For cultured cells, once the cultured pulp cells got 70%-80% confluent, first, they were washed x3 with PBS to remove media then they were trypsinized using 0.25 Trypsin/EDTA for 5 min at 37° c. After confirmation of dissociation, sample was processed as explained with the fresh pulp tissue.

2.6.3 Staining protocol

The cell pellet was resuspended in FACS blocking buffer (10%FBS) at density of 5×10^3 cells/ml.

2.6.3.1 Conjugated antibodies

0.5 µg of antibody was added to cell suspension and incubated in dark at 4°C for 30 min. Then the antibody was washed x3 using the blocking buffer and centrifuged as mentioned above to get the cell pellet.

2.6.3.2 Unconjugated antibodies

0.5-10 µg of primary antibody was added to the cell suspension and incubated in dark at 4°C for 30 min. Then the primary antibody was washed x3 using the blocking buffer and centrifuged as mentioned above to get the cell pellet. The cell pellet was resuspended again in blocking buffer and the 0.5-10 µg secondary antibody was added and incubated for the same time and conditions for primary antibody. Then the secondary antibody was washed x3 using the blocking buffer and centrifuged as mentioned to get the cell pellet.

2.6.3.3 Sample fixation

Cell pellet then resuspended in 4% PFA for 10-15 min at Room Temperature (RT) then washed with PBS and centrifuged to get the cell pellet which was resuspended and kept in x1 PBS until acquisition and analysis.

2.6.4 Data acquisition and analysis

- Data was acquired using BD FACSCanto II flow cytometer.
- Data was analyzed using BD FACSDiva version 6.1.3
- Each flow cytometry analysis was replicated three times and in each passage, the mean percentages of the cell expressing the particular antigens were calculated and compared to other passages.
- Positive expression was defined as the level of fluorescence >99% of the corresponding isotype-matched control antibodies.

2.7 Magnetic Activated-Cell Sorting (MACS).

To sort out Thy-1⁺ cells, MACS was performed as follow

2.7.1 Antibodies

Mouse monoclonal to Thy-1 CD 90 conjugated to Fluorescein Isothiocyanate (FITC) as used in flow cytometry (2.6.1)

2.7.2 Sample preparation

As mentioned in section 2.6 above

2.7.3 Antibody staining

As mentioned in section 2.6.3.1 above

2.7.4 Magnetic labelling

The magnetic labelling procedure was performed according to Miltenyi Biotec MACS separator system's instructions. Briefly, the cell pellet was resuspended in MACS buffer (0.5%BSA, 2mMEDTA in PBS). A total of ($6 - 8 \times 10^6$) cells from primary cells were incubated with 10 μ l Anti-FITC microbeads (Miltenyi) for every 10^7 of cells for 15 minutes at 4°C. Then sample was then washed to remove excess beads and cells were centrifuged at 1200 rcf for 5 minutes. Formed cell pellet then was resuspended in 500 μ l of MACS buffer and now it is ready for magnetic separation.

2.7.5 Magnetic Separation

Magnetic separation procedure was performed according to Miltenyi Biotec MACS separator system's instructions. At this stage, the positive cells were magnetically labelled with Anti-FITC microbeads. Prior to sorting out the cells, the MACS separator columns must prepared by rinsing with appropriate volume of MACS buffer. Then the cell suspension was applied onto the column. The unlabelled cells passed through and were collected while the magnetically labelled cells were eluted out by the MACS buffer after removing the column from the magnetic separator. To test the efficiency of the magnetic

cell sorting protocol, separated cells were labelled with Thy-1 antibody and go for flow cytometry analysis as described before.

2.8 Microarray samples preparation

Samples were prepared by *Dr Puangwan Lapthanasupkul* and *Dr Jifan Feng*.

Mouse mandibular incisors dental pulp tissues were collected as described in 6.3.9). RNA extraction was done according to manufacturer's instructions using RNeasy mini kit (Qiagen). The previous cell pellets were disrupted and homogenized in a denaturing buffer RLT that contains guanidine thiocyanate, which instantly inactivates RNases to ensure the purification of intact RNA. 70% Ethanol was then added to provide appropriate binding conditions to the silica based membranes of the spin columns. Buffers RW1 and RPE were then added to the columns which were then spun in the microcentrifuge at 10,000 rpm. RNA was eventually eluted in 20-50µl of RNase-free water and sent for Microarray using GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Inc) and the data was analysed using R statistics (free software, v.2.13.2).

2.9 Obtaining embryonic and neonatal mouse tissues

All animal experiments were approved by the UK Home Office. Unless otherwise stated, colonies of wild type and transgenic mice were maintained in a homozygous state with the assistance of Mr. Alex Huhn. Mice carrying the Thy-1 Cre, Ptc-1^{fl/fl}; CreERTm and Polaris^{fl/fl}; CreERTm transgene were identified by performing a genotyping polymerase chain reaction (PCR) that amplified a *Cre* and CreERTm specific product. Genotyping was performed by *Miss Livia Katonova* and *Mr. Andrew Donkin*. Male and female mice were mated overnight and vaginal plugs were checked using a probe the following day. The day on which the vaginal plug was discovered was used to determine the embryonic stage and was designated as embryonic day 0.5 (E0.5). Collection of embryonic and neonatal

tissue was carried out according to the Home office schedule one specification. Cervical dislocation was used to sacrifice pregnant females to obtain embryos at specific time points. Embryos were removed by caesarean section and collected in ice-cold 1XPBS. For postnatal pups, the day a litter was found was designated as postnatal day 0 (P0). Pups were sacrificed by cervical dislocation followed by decapitation. After excess blood was blotted on a tissue paper, pup heads were collected in ice-cold 1XPBS.

2.10 Generation of Ptc-1^{fl/fl};CreERTm and Polaris^{fl/fl};CreERTm transgenic mice

2.10.1 Generation of transgenic mice

Ptc-1 and Polaris double floxed mice were gifts from Prof Martin Cobourne and Dr Atsushi Ohazma (Roy and Ingham, 2002) respectively. To generate the Ptc-1^{fl/fl} CreERTm transgenic mouse line, A ptc^{fl/fl};CreER^{Tm+/+} mouse was crossed with a ptc^{+/+};CreER^{Tm+/-} mouse to produce the first generation with a genotype ptc-1^{+/fl};CreERTm^{+/-}. They were then bred together to give the second generation of ptc-1^{fl/fl};CreERTm^{+/-} mice. To maintain this line, these mice were mated with ptc-1^{fl/fl} mice, with 50% carrying the CreERTm as well.

Polaris^{fl/fl};CreERTm mouse line was produced and maintained in similar way.

2.10.2 Tamoxifen Administration

For Conditional deletion of Ptc-1 and Polaris genes in Ptc-1^{fl/fl};CreERTm and Polaris^{fl/fl};CreERTm bitransgenic mice respectively, 4 weeks mice were given by oral gavage once daily of 4 mg/ 30g body weight (Burns et al., 2007) of Tamoxifen (Tam) (Sigma-Aldrich) (200µl of 20mg/ml Tamoxifen in corn oil solution) for five successive days. One week after the completion of the Tamoxifen treatment, mice were collected.

2.10.3 Cre transgenic mice

One of the methods of lineage tracing is genetic recombination. Genetic recombination in mice is usually performed using the Cre-loxP system in which Cre recombinase is expressed under the control of a tissue- or cell-specific promoter in one mouse line. That line is crossed with a second mouse line in which a reporter is flanked by a loxP-STOP-loxP (“floxed” STOP) sequence. In animals expressing both constructs, Cre specifically activates the reporter in cells that express the promoter, by excising the STOP sequence (Figure 2-1).

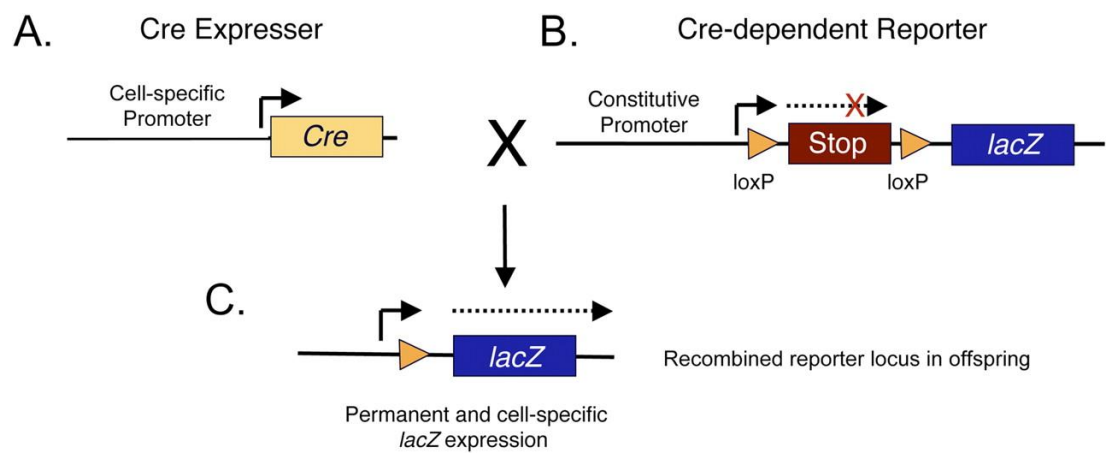


Figure 2-1: Schematic of the Cre/loxP lineage tracing methodology. Transgenic mice expressing the Cre recombinase under the control of a cell-specific promoter (A) are crossed to reporter mice (B) in which a reporter gene (e.g., *lacZ* in R26R mice) is separated from a constitutively active promoter by transcriptional stop sequences that are flanked by loxP sites, the target sequences recognized by Cre recombinase. Only cells of cell-specific Cre X loxP-*lacZ* offspring that express Cre undergo DNA excision of the stop sequences, resulting in transcription of the reporter gene (C) (Lounev et al., 2009).

2.10.4 CreERT^m transgenic mice

The inducible Cre recombinases are fusion proteins containing the original Cre recombinase and a specific ligand-binding domain (LBD). A requirement for temporal control of Cre-mediated recombination is that the inducible Cre recombinase should be activated only by an exogenous ligand introduced at a chosen time. To achieve that, the

engineered Cre recombinase is fused with mutated hormone LBDs. In such cases, synthetic ligands bind the modified Cre recombinase while the endogenous ligands can't. Binding of the ligand is thought to produce conformational changes of the fusion protein and/or changes in the intracellular localization associated with targeting of the recombinase to the nucleus (Brian 1998; Metzger and Feil 1999)

The most common strategy for temporally regulating Cre activity has been to generate Cre fusion proteins with a Tamoxifen-responsive Estrogen Receptor ligand binding domain from human gene as ERT, (Brocard, et al. 1997; Feil, et al. 1996) and ER T2 , (Feil, et al. 1997) or mouse gene as ER TM , (Hayashi and McMahon 2002). CreER is sequestered in the cytoplasm by heat shock protein 90 (Hsp 90). The administration of Tamoxifen confers a conformational change on CreER that releases it from Hsp 90 and allows it to translocate to the nucleus, where it induces recombination between loxP sites leading to the deletion of the floxed gene (Figure 2-2).

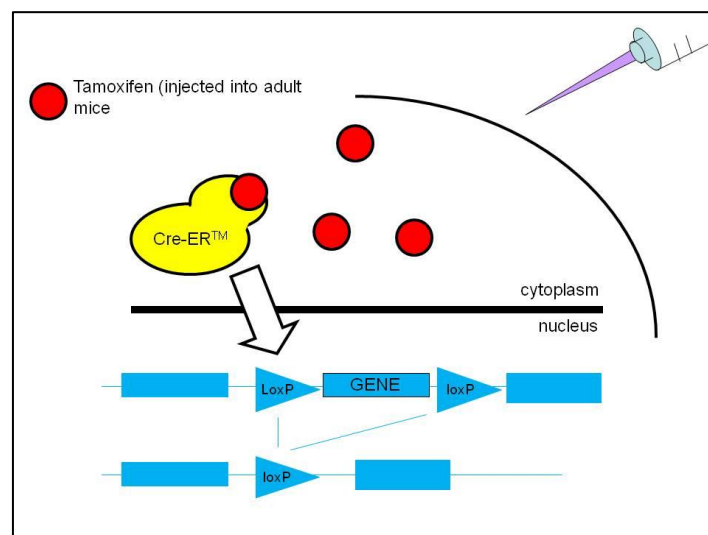


Figure 2-2: Effect of Tamoxifen when injected into inducible CreER adult mice. With Tamoxifen injection, CreER is translocated into the nucleus where recombination occurs between Cre and Loxp sites leading to the deletion of the floxed gene. (Adapted from online presentation).

2.11 Tissue Processing

2.11.1 Fixation, Decalcification and Dehydration of Tissues

Heads of mouse embryos or jaws of post-natal mice were dissected in cold 1X nuclease-free PBS. The tissues were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. For E13.5 or older embryonic heads, fixation was prolonged to 48-72 hours. Mouse tissues older than E16.5 were decalcified in 4% or 12.5% EDTA pH8.0 containing 1% PFA for 1 - 3 weeks at room temperature on a shaker, depending on the developmental stages of the embryos/mice. Adult mouse tissues were decalcified for 3 - 6 weeks with 18% EDTA at room temperature on a shaker. All decalcifying solutions were freshly prepared and changed every day. After fixation or decalcification, tissues were thoroughly washed with 1X nuclease-free PBS to eliminate residual PFA or formic acid and then dehydrated in ascending ethanol (VWR BDH Prolab) solutions (30%, 50%, 70%, 85%, 95%, and 100%). The duration of each step was determined by the size and age of the sample, as listed in the (Table 2-7) below.

Table 2-7: Dehydration time in ethanol according to the specimen.

Sample	Ascending Ethanol Solutions
Embryo head (E12.5- E14.5)	1-3 hr per change
Embryo head (E15.5- E16.5)	4-6 hr per change
Embryo head (E17.5- E18.5)	Overnight – 24 hr per change
Postnatal/Adult mouse jaw	6 hr – overnight per change

2.11.2 Paraffin Embedding

To allow the replacement of ethanol by paraffin wax, after final dehydration in 100% ethanol, the tissues were incubated in several changes of histoclear (national diagnostics). Tissue samples were then incubated in histoclear:paraffin mix (in 1:1 ratio) at 60°C, following by several consecutive wax changes. The detailed duration of each step for different tissues is specified in (Table 2-8). After the long incubation period in

paraffin wax (Solmedia), whole heads were embedded at a specific orientation (frontal or sagittal) using metal moulds. Wax blocks were stored at 4°C until sectioning.

Table 2-8: Duration of each embedding step according to the developmental stage and size of the specimen.

Tissues	1,2,3,tetahydron aphthaline	1,2,3,tetahydron aphthaline:Wax (1:1)	Wax	Vacuum Embedding
E12.5-E14.5 (Head)	20 min x 6	30 min	1 hr x 6	30 min – 1 hr
E15.5-E16.5 (Head)	30 min x 6	30 min	1 hr x 6 + overnight	1 hr
E17.5-E18.5 (Head)	1 hr x 5	30 min	1 hr x 6 + overnight	1 hr
Postnatal/Adult (Jaw)	1 hr x 6 + overnight	30 min	1hr x 6-12 + overnight	1 hr

2.11.3 Tissue Sectioning

Wax blocks were firstly trimmed to remove excess wax. The samples were then sectioned to produce wax ribbons of 5-7µm in thickness, using a microtome (Leica RM2245). Consecutive sections were then mounted on different glass slides (SuperFrost®Plus, VWR™) to produce a series of slides containing a similar set of serial sections.

2.11.4 Haematoxylin and Eosin Staining (H&E)

H&E staining was used to view general cell morphology. Haematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. One set of sections was deparaffinised with two 10 minute histoclear washes and rehydrated through a graded series of ethanol washes (100%, 90%, 70% and 50%) for 2 minutes each. Sections were then washed for ten minutes in distilled water and submerged in Erhlich's Haematoxylin (Solemedia) for 10 minutes. Excess haematoxylin was removed by washing the samples for another 10 minutes under running

water. Prior to differentiation, sections were rinsed in distilled water and then submerged in acid alcohol (0.5% HCl 35% alcohol) for 15 seconds. Subsequently, the sections were stained with 0.5% aqueous Eosin (Riedel-de-Haen,32617) for 2 minutes, washed in distilled water and dehydrated through a series of two minute ethanol washes (70%, 90% and two 100%). Sections were air-dried for up to 1 hour before being covered with coverslips using DePex under the fume hood. Sections were viewed in light-field using a Zeiss microscope (Axioskope 2 plus) and captured with an AxioCam HRC (Zeiss) using Axiovision software.

2.12 Staining for β -galactosidase (LacZ) activity

2.12.1 Whole Mount β -galactosidase Staining

2.12.1.1 Sample collections, fixation and staining

Transgenic mice carrying a LacZ reporter were processed through LacZ staining protocol prior to histology analysis. Samples were carefully dissected in cold PBS: embryonic heads were cut in half to allow better penetration of both the fixative and the staining solution; postnatal teeth were carefully dissected from the mandibles and maxillae to provide better penetration access for the solution. Dissected tissues were placed immediately in a fixative solution made of 1% PFA and 0.2% glutaraldehyde (Merk Millipore) in PBS at room temperature. Fixation time also varied depending on the tissue and is detailed in (Table 2-9). Alternatively, some postnatal samples were fixed in 4% PFA at 4°C for 1 hour for better immunohistochemistry reaction.

Table 2-9: Fixation time for lacZ staining.

Sample Stage	Incubation Time (minutes)
E14.5	25
E17.5	40
Postnatal	60-O/N

The fixed tissue was then washed in 1XPBS and incubated with X-gal staining solution (Table 2-10) at 37 °C and protected from light. Adequate reaction colour (blue) developed usually after approximately 24-48 hours after initial incubation. After staining, samples were washed 3 times in 1XPBS for 10 minutes each or longer, the stained samples were post-fixed in 4% PFA for at least one hour in room temperature for histological processing.

Table 2-10: Components of lacZ staining solution

Components	Concentration
Tris HCl pH 7.3	10mM
Sodium deoxycholate(Sigma-Aldrich)	0.005%
IGEPAL(Sigma Adrich)	0.01%
K ₃ Fe(CN) ₆ (VWR BDH Prolabo)	5mM
K ₄ Fe(CN) ₆ (VWR BDH Prolabo)	5mM
MgCl ₂ (Fisher)	2mM
X-Gal (Fermentas)	0.8 mg/ml
1x PBS	up to final volume

2.12.1.2 Processing X-gal Stained tissues for sectioning

After post-fixation, X-gal Stained samples were washed 3 times in 1XPBS for 10 minutes each or longer. Mouse tissues older than E16.5 were decalcified as previously described in (2.11.1). Samples were dehydrated through a graded series of methanol solutions (30%, 50%, 70%, 85%, 95% and 100%) to minimise de-staining. The duration for each wash step depended upon the age of the sample (Table 2-11).

Table 2-11 Methanol dehydration time for X-gal stained sample (per step)

Sample Stage	Incubation Time(minutes)
E14.5	35
E17.5	45
Newborn	60
Adult	180

After the sample was completely dehydrated, 100% methanol was replaced by two changes of isopropanol (Acros Organics) for 15 minutes; the samples were then placed in tetrahydronaphthalene (THN) (Sigma-Aldrich) at room temperature till saturated and moved to 60°C for 15 minutes. Afterwards samples were placed in a 1:1 mixture of THN: paraffin wax at 60°C for 15 minutes. Finally, embryonic samples younger than E13.5 were washed at least four times (each wash lasting 1hr) in paraffin wax at 60°C before embedding. Samples that were E14.5 or older were washed for at least 5 times in wax for one hour each followed by an additional overnight incubation. Wax embedded samples were sectioned and mounted as described in section (2.11.3)

2.12.1.3 Counterstaining of X-gal Stained Sections

Sections from transgenic mice that leave nuclei X-gal stained were counterstained with 0.2% Nuclear Fast Red (Sigma Aldrich) to allow the identification of unstained structures. Sections were de-waxed in histoclear for 2x 10 minutes and rehydrated briefly in descending ethanol solutions 100%, 90%, 70% and 50% for 1 minute each. The slides were then stained in Alcoholic Eosin (0.25%) from 45 seconds to 2 minutes and excess staining was washed away in dH₂O. Sections were then dehydrated in ascending ethanol 70%, 90% and 100% ethanol for 1 minute each. The slides were then air-dried and mounted with DePex.

2.12.2 β -galactosidase staining for frozen sections

Sections were kindly cut by *Dr Simone caixeta*.

2.12.2.1 Fixation, embedding and sectioning

Samples were collected and washed in 1XPBS the same way for whole mount LacZ staining samples. Then they were placed in the fixing solution (Table 2-12) at 4°C for different times according to the age of the sample (45 minutes E14.5, 1 hour E17.5).

Table 2-12 Fixing solution for frozen sections

Component	Volume	Final concentration
Glutaraldehyde	100 μ l	0.2%
1M MgCl	100 μ l	2mM
Add H ₂ O to	50ml	

The following day, samples were washed in 1XPBS three times for 15 minutes each to remove the remaining fixing solution completely. Samples were then dehydrated in sucrose solution (Table 2-13) at 4°C overnight.

Table 2-13 Sucrose dehydrating solution

Component	Volume
Sucrose 60% (SIGMA,S0389)	12.5 μ l
1M MgCl	100 μ l
Add 1X PBS up to	50 ml

After that, samples were further dehydrated in sucrose dehydrating solution II (Table 2-14) which is a 1:1 ratio of sucrose: O.C.T (optimal cutting temperature) compound (VWR BDH prolabo) on a shaker for 90 minutes at room temperature followed by 4°C overnight. Finally samples were embedded in O.C.T in a frontal

orientation on a box containing dry ice and 100% Ethanol. When it was completely white and hard, it was transferred to -80°C freezer.

Table 2-14 Sucrose dehydrating solution II

Component	volume
Sucrose 60%	25ml
OCT	25ml
1M MgCl	100μl
Add 1X PBS up to	50 ml

The frozen samples were then cryo-sectioned using the cryostat into thickness of 15μm in a frontal plane. The sections were then mounted on tissue adhering slides and stored at -20°C.

2.12.2.2 Slides Staining and counterstaining

For X-gal staining, cryosections were allowed to dry at room temperature for at least 4 hours then place the slides on the glass Coplin jar. Slides were then post-fixed in fixative solution for exactly 30 minutes on ice. Slides were promptly washed for 10 minutes on ice with 1XPBS containing 2mM MgCl₂ followed by detergent rinse solution (Table 2-15) for 10 minutes.

Table 2-15 Detergent rinse solution

Component	Volume
1M MgCl	100μl
Sodium Deoxycholate 5%	100μl
Nonidet P-40 (Igepal)	250μl
Add 1XPBS up to	50 ml

Sections were then placed in stain solution (Table 2-16) for 24 hours at 37°C in the dark. Following the staining reaction, slides were washed twice in 1XPBS for 15 minutes and then post fixed in 3.7% formaldehyde for 1 hour at room temperature. To counterstain X-gal stained sections, slides were rinsed in water and then washed for 20 seconds in nuclear fast red. Washing the sections in 30%, 50% and 70% ethanol for 10 seconds each then dehydrated them. Slides were then stained in Eosin for 1 minute before finally washing them in 90% ethanol and xylene for 10 seconds. Slides were immediately mounted with DePex.

Table 2-16 staining solution

Component	Volume
1M MgCl	100µl
Sodium Deoxycholate 5%	100µl
Nonidet P-40 (Igepal)	250µl
K3	5ml
K4	5ml
Xgal(50mg/ml)	1ml
Add 1X PBS up to	50 l

2.13 Micro Computerised Tomography (Micro-CT) Analysis

Mouse heads were dehydrated in a graded ethanol series (30%, 50% and 70%) and then scanned using a GE Locus SP micro CT scanner. Mouse heads were immobilised using ultrasound gel and cotton gauze and scanned to produce 14 µm voxel size volumes. After scanning, Explore Microview software programme (GE) was used for visualization and analysis. In this study, mouse teeth were characterised by generating three dimensional reconstructions and three dimensional isosurfaces of mouse teeth were then produced. For measurement of mouse incisor length, the locations of incisal end and

apical end of each incisor were identified on the microCT planes as three coordinates (x,y,z). The distance between these two points ((x₁,y₁,z₁) and (x₂,y₂,z₂)), representing the incisor length, was then calculated using the formula derived from the three dimensional version of the Pythagorean theorem (Distance = $\sqrt{(x_2-x_1)^2+(y_2-y_1)^2+(z_2-z_1)^2}$).

2.14 Statistical analysis

All experiments were repeated at least three times, and the data were expressed as the mean \pm SD in all figures. Student's *t* test analysis was performed using Microsoft Excel 2007. Differences between experimental and control groups were regarded as statistically significant when *P* < 0.05. One-way analysis of variance (ANOVA) test and post hoc test, Bonferroni correction were performed using GraphPadPrism software (v 5.01).

2.15 Organ Culture

CD-1 pups were sacrificed with cervical dislocation. Incisor tooth germs were carefully dissected from the mandibles of mice at desired stages of development under a stereomicroscope. For incisor explants, the whole incisor was placed onto 0.1 μ m pore size filter. Explants were cultured at the medium-gas interface in an organ culture dish (BD Falcon) on nitrocellulose filters (Millipore) supported by metal grids in a humidified atmosphere of 5% CO₂ at 37 °C. The explants were cultured in the medium containing Alpha Modified Eagle Medium supplemented with 20% FBS, 20 units/ml penicillin/streptomycin. For the signaling inhibitor experiments, inhibitors including cyclopamine (20 μ M) (Toronto Research Chemicals, Ca) and SAG (200nm) (Merk Millipore) were added to the culture medium. Explants were cultured *in vitro* with SAG and cyclopamine for 24-48 hours respectively. Explants were then fixed with 4% PFA overnight and processed for whole-mount *in situ* hybridization.

2.16 Immunohistochemistry

The sections were de-paraffinized in histoclear for 10 minutes twice and rehydrated through decreasing concentrations of ethanol series (100%, 95%,90%,70%,50% and 30%) followed by 1X PBS. After rehydration, permeabilization was performed by incubating the sections in 1% Triton X100 in 1X PBS for 30 minutes at room temperature. For Antigen retrieval, the sections were microwaved in citrate buffer pH 6.0 or Tris-HCl pH 9.0 for 30 minutes at 95 °C and then cooled down for at least 30 minutes at room temperature. After antigen retrieval, non specific bindings were blocked by incubation with blocking buffer for 1 hour at room temperature then sections were incubated in a 1/100 dilution of primary antibody (Thy-1, CD133 and CD146) in blocking buffer (10% blocking powder in 1X PBS) overnight at 4°C in a humidified chamber. Details of antibodies used in immunohistochemistry are listed in table (2-17)

Table 2-17: Antibodies and optimal dilutions used in immunohistochemistry

Gene	Dilution	Gene Localization	Manufacture
Mouse monoclonal to Thy-1 CD 90 conjugated to Fluorescein Isothiocyanate (FITC)	1:100	Cell Surface	Abcam 62009
Mouse monoclonal [P1H12] to CD146 conjugated to Phycoerythrin (PE)	1:70	Cell surface	Abcam 75769
Rat anti mouse monoclonal to CD133 (Prominin-1) conjugated with Allophycocyanin (APC)	1:700	Cell surface	e- bioscience
Purified rabbit polyclonal to Patched / PTC	1:50	Cell surface	Abcam 53715
Mouse IgG2bk isotype control FITC	1:100	Cell surface	e-bioscience

The following day, the sections were washed in 1X PBS 1 minute three times and then incubated in a 1/300 dilution of secondary antibody conjugated with biotin in blocking buffer for 1 hour at room temperature in a humidified chamber. To perform peroxidise (Vector labs) visualization for the biotin conjugated antibody, the sections were incubated in ABC solution (Vectastain kit, Vector labs) for 1 hour at room temperature

and then washed in 1X PBS. The colour reaction was then developed by applying DAB solution (0.5 mg/ml DAB and 0.1% H₂O) onto the sections. When the colour reaction was satisfactory, the reaction was stopped by rinsing with H₂O for 5-10 minutes and the sections were then counterstained with hematoxylin (Fluka) for 2 minutes. Following the counterstain, the sections were gradually dehydrated and mounted with coverslips using vectamount permanent mounting medium (Vector labs).

2.17 Osteogenic differentiation of incisor dental pulp stem cells.

2.17.1 Induction

Mouse incisor dental pulp cells were expanded in culture and plated in 12 well plate. Dental pulp cells were induced at two different passages; passage number 1 and passage number 4. After 24 hour, Cells were induced for osteogenic differentiation using commercial osteogenic media (Gibco, STEMPRO Osteogenesis Differentiation Kit) and α MEM was used as the growing media for the control. Osteogenic media as well as control media were changed twice a week. Thy-1 expression was investigated at the same passages just before plating cells for differentiation.

2.17.2 Detection

After 21 days, cells were fixed with ice cold 4% PFA for 10 minutes. After washing PFA, mineralization was assayed using 2% Alizarin red staining (pH =5.2) (Sigma-Aldrich) for 10 minutes at room temperature. Excess staining was eliminated by washing in PBS for 5 minutes. Plates were then scanned using a modified scanner using preview program (Apple software).

Chapter 3 :Mesenchymal Stem cells in the mouse incisor

3.1 Introduction

Mouse incisors differ from molars and all human teeth in that they erupt continuously throughout the life of the animal (Harada et al., 1999). This continuous growth and eruption is related to the presence of the cervical loop area, which is thought to be the reservoir for the cells producing enamel and dentin of the erupting tooth (Harada et al., 1999). In these incisors, the epithelial stem cells are located mainly within the labial cervical loop while no progeny of the stem cells are found in the lingual cervical loop (Wang et al., 2007). It has been suggested that MSCs reside inside the dental pulp in two locations; the perivascular area and the apical end of the pulp mesenchyme (Feng et al., 2011). From a previous mouse dental pulp stem cell microarray comparing stem cell gene expression between incisor and molar dental pulp, the expression of several stem cell genes-such as *Crabp1*, *Hus-1*, *Ikaros* and *Thy-1*, found to be up regulated in the incisor, was investigated (Mantesso and Sharpe, unpublished data, 2008). A second microarray experiment, comparing gene expression between the cervical loop area and the body of mouse incisor dental pulp tissues was performed (Lapthanasupkul and Feng, unpublished data, 2009), and the common genes between these two microarrays were identified. *Thy-1* is a mesenchymal stem/progenitor cell marker (da Silva Meirelles et al., 2008, Nakamura et al., 2010). *Thy-1* was used as a marker for mouse haematopoietic stem cells (Petersen, et al. 1998), and is expressed in keratinocyte stem/progenitor cells (Nakamura, et al. 2006). Recently, *Thy-1* was reported to be expressed in the subodontoblastic zone in rat incisors (Hosoya, et al. 2012).

This chapter will show how Thy-1 was used to define a mesenchymal stem cell niche within the mouse incisor dental pulp, also how Thy-1 expression in dental pulp tissues and cultured dental pulp stem cells was investigated. Moreover, dental pulp mesenchymal stem cells were further characterized using other stem cell markers CD146 (Shi and Gronthos, 2003) and CD133 (Kania et al., 2005) together with Thy-1.

3.2 Results

3.2.1 Mesenchymal stem cells in the dental pulp of mouse incisors

3.2.1.1 Mesenchymal stem cells are located in the apical mesenchyme inside the mouse incisor dental pulp

Mouse incisors are different from molars, since they are continuously growing due to the presence of the cervical loop area which harbours stem cells required for growth (Harada et al., 1999). A previous stem cell microarray had been carried out to compare stem cell gene expression between incisor (I) and molar (M) dental pulp cultured cells. Few genes were up-regulated in the incisor dental pulp when compared to their expression in molars. From these genes, *Crabp-1*, *Hus-1*, *Ikaros* and *Thy-1* were selected for further investigation. Except for *Crabp1*, these genes are not previously identified to be expressed at any time during mouse incisor development and so they do not act in tooth patterning and differentiation (<http://bite-it.helsinki.fi>).

To locate gene expression in the mouse incisor dental pulp tissue, whole mount *in situ* hybridization was performed for postnatal day 5 (PN5) mouse incisors. Interestingly, all of these genes were expressed in the cervical loop area where the mesenchymal stem cell niche is believed to be located (Feng et al., 2011). *Crabp-1* was intensely expressed in the mesenchymal tissue adjacent to the labial cervical loop, but it had weak expression in the mesenchymal tissue next to the lingual cervical loop and the tissue between the two cervical loops (Figure 3-1, A, A' and A''). *Hus-1* expression was more intense in the mesenchymal tissue related to and in between the labial and lingual cervical loops (Figure 3-1, B, B'). Vibratome sections showed that the mesenchymal tissue next to the labial cervical loop had no *Hus-1* expression (Figure 3-1, B''). *Ikaros* expression was similar to *Crabp-1* but with

slightly higher expression in the mesenchymal tissue between labial and lingual cervical loops (Figure 3-1, C, C' and C''). Thy-1 expression was found in the mesenchymal tissue adjacent to the labial cervical loop in addition to few of the basal dental epithelial cells (Figure 3-1, D and D'). Thy-1 expression was correlated with the blood vessels in the dental follicle (Black arrow inset, figure 3-1, D'') and inside the dental pulp (White arrows inset, figure 3-1, D'') Also, its expression adjacent to the lingual cervical loop or other parts of the mesenchymal tissue was weak.

To view these gene expression patterns in three-dimensions (3D), Optical Projection Tomography (OPT) was used. OPT is a relatively new technique for 3D imaging of small biological tissues (Sharpe et al., 2002) that is particularly useful for reconstructing vertebrate embryos and for examining the 3D anatomy of developing organs. In this experiment, OPT showed the 3D mRNA expression after detecting it using *in situ* hybridization. Crabp-1, Hus-1, Ikaros and Thy-1 expression was all detected in the cervical area of the mouse incisor (Movies A, B, C and D respectively, included in the enclosed DVD). Representative images of the samples from the movie are shown in (Figure 3-2).

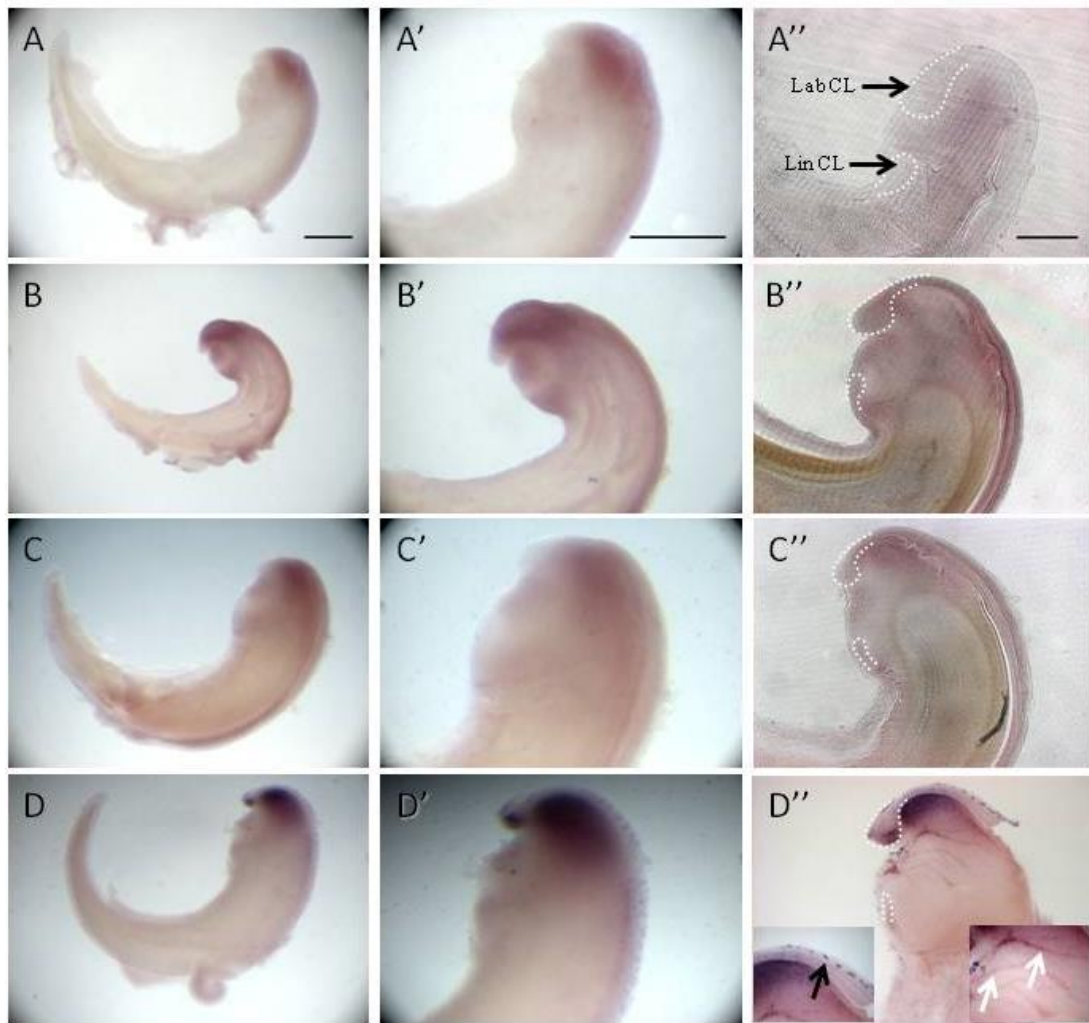


Figure 3-1: Expression of incisor up-regulated genes in postnatal day 5 (PN5) mouse mandibular incisors by whole mount *in situ* hybridization (Sagittal sections). Crabp-1 is expressed in the apical end of developing incisor tooth germ (A, A'), (A'') A vibratome section shows strong expression of Crabp-1 in the mesenchymal tissue adjacent to the labial cervical loop, while its expression near the lingual cervical loop and mesenchymal tissue between them is faint. (B, B') Hus-1 is expressed in the mesenchyme adjacent to the labial and lingual cervical loops and the mesenchymal tissue between them. (B'') A vibratome sections showing the mesenchymal tissue next to the labial cervical loop is devoid from Hus-1 expression. Expression of Ikaros is limited to the mesenchyme next to the labial and lingual cervical loops in addition to a part of the mesenchyme between them (C, C', C''). Thy-1 expression was the strongest (D, D') with its expression intensified in the mesenchymal tissue adjacent to the labial cervical loop and its tip. (D'') A vibratome section shows Thy-1 expression in the blood vessels of the dental follicle (Black arrow inset) and dental pulp (White arrows inset). White dots outline the labial and lingual cervical loops. (Lab) labial, (Lin) lingual, (CL) cervical loop. Scale bar=500µm, and =250 µm in insets

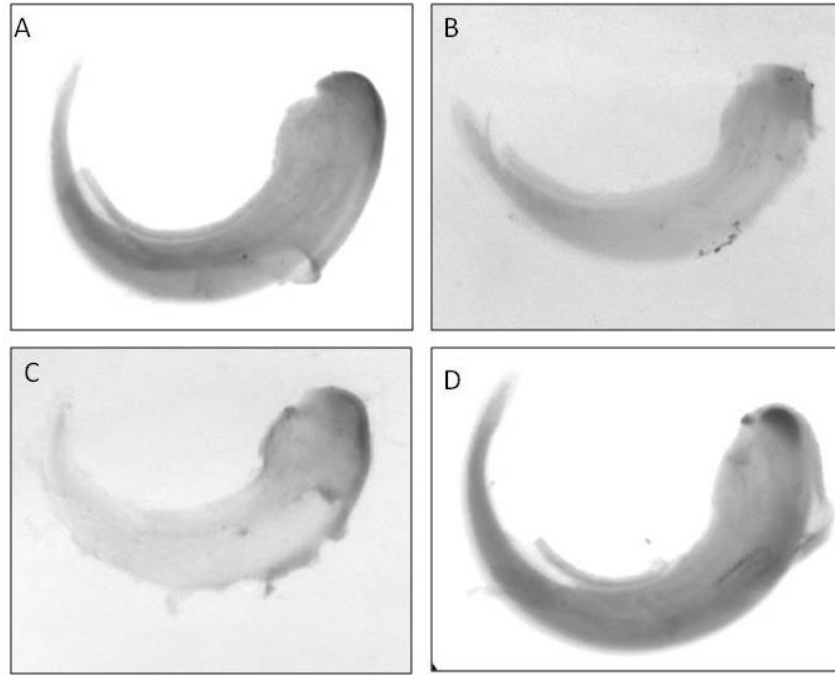


Figure 3-2: Still images of PN5 mouse mandibular incisor 3D construction after *in situ* hybridization. (A) Crabp-1,(B) Hus-1,(C) Ikaros and (D) Thy-1 were expressed in the apical area of the mouse incisor where the mesenchymal stem cells are located.

In summary, Crabp-1, Hus-1, Ikaros and Thy-1 were expressed in the cervical area where mesenchymal stem cells are located.

To find genes that are specific to the cervical loop area, another microarray comparing gene expression between the body and cervical loop area of mouse incisor was performed.

3.2.1.2 Microarray comparing gene expression between the body and cervical loop of mouse incisor dental pulp tissue

Since the cervical loop area has the stem cell populations required for the continuous growth of the mouse incisor (Harada et al., 1999), a microarray screen comparing gene expression between the cervical loop and the body of the mouse incisor dental pulp tissues was performed. In the Affymetrix GeneChip, around 45,000 probe sets

analyze the expression level of over 39,000 transcripts and variants from over 34,000 well characterized mouse genes. We employed GeneChip analysis to compare the expression levels of approximately 45101 kinds of transcripts in 6 samples; PN5 incisor pulp body (Body) (n=3) and PN5 incisor pulp cervical loop (CL) area (n=3). Altogether, 6 GeneChips were used to examine the gene expression profile of two different samples. Differential expression was stated if a distinct transcript met the corrected P-value of 0.05 or less. We identified 1072 differentially expressed genes, of which 699 genes were up-regulated and 373 genes were down-regulated in CL/Body respectively. Only 453 transcripts showed more than 2 log₂ Fold Change (FC) in CL/Body. Of these transcripts, 432 had sufficient annotation for further investigation.

The Heatmap, comparing the 354 genes most differentially expressed in the body and cervical loop is shown in (Figure 3-3).

Comparing the up-regulated genes in this microarray with those of the previous stem cell microarray revealed that only three genes were in common; *Dlx5*, *Thy1*, and *Thbs1*. The expression site of these genes was very interesting as they were either up-regulated in the incisor dental pulp which is believed to have a stem cell population necessary for its continuous growth, or they were up-regulated in the cervical loop area which is a well known stem cell site within the mouse incisor. *Dlx5* was up-regulated in the dental pulp of molar and cervical loop, while *Thbs-1* was up-regulated in the dental pulp of molar and the body of incisor and finally *Thy-1* was up-regulated in the dental pulp of incisor and the cervical loop (Figure 3-4).

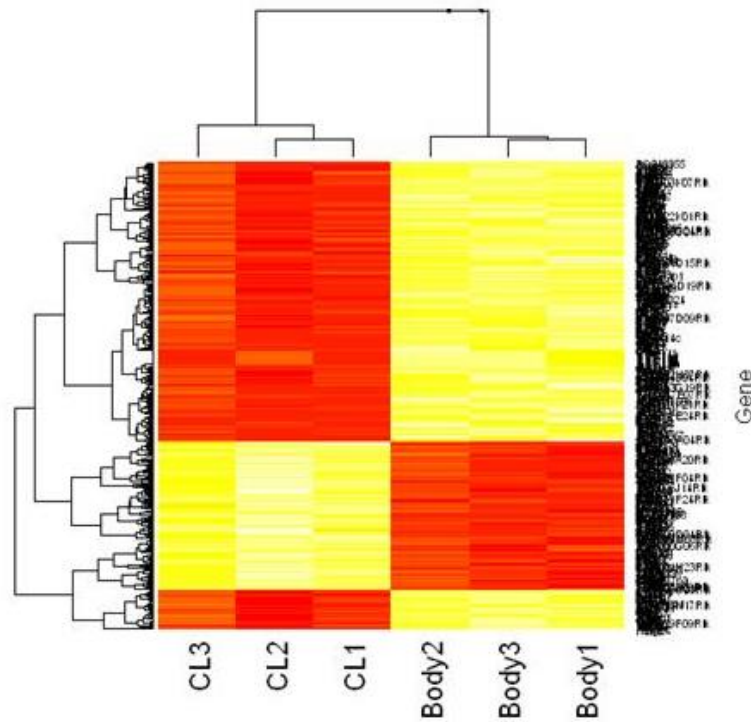


Figure 3-3: Heatmap comparing gene expression between the incisor body (Body) and the cervical loop (CL) pulp tissues. Many genes were up-regulating (Red area) in the cervical loop samples (CL1, CL2 and CL3) when compared to their expression (yellow area) in the body pulp tissue (Body1, Body2 and Body3). Also fewer genes were up-regulated in the body samples compared to the cervical loop ones. Rows represent genes, and columns represent the samples. Genes that fall into one cluster (vertical axis) have similar behaviour in the experiments. Genes that fall into one cluster (horizontal axis) share the same category. Expression intensities are represented by red and yellow, for high and low intensities, respectively.

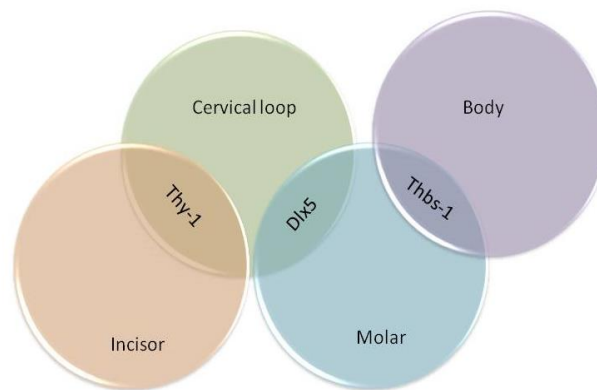


Figure 3-4: The illustration shows the common genes in stem cell and Body/CL microarrays. *Thy-1* was found up-regulated in incisor pulp tissues when compared to molar pulp tissue (Analysis of stem cell genes – Microarray 1) and in the cervical loop when the genetic expression of this area was compared to incisor body areas (Analysis of multigenes – Microarray 2), while *Dlx5* was up regulated in molar pulp tissues (Microarray 1) and the incisor cervical loop (Microarray 2). On the other side, *Thbs-1* was up regulated in molar pulp tissues (Microarray 1) and the incisor body (Microarray 2).

Interestingly, Thy-1 expression was up-regulated in both incisor dental pulp and the cervical loop area (which is the common site for stem cells inside the mouse incisor) compared to its expression in molar dental pulp and the body of the incisor. Added to that, Thy-1 is a well known mesenchymal stem cells marker (Horwitz et al., 2005). For these reasons Thy-1 was selected for further investigation in this study.

3.2.2 Validation of Thy-1 expression in Body/CL Microarray results

The increase in Thy-1 expression in the cervical loop area was validated by flow cytometry. Fresh PN5 mouse mandibular incisor dental pulp was digested and stained by Thy-1 antibody. The Thy-1⁺ population was shown to be highly enriched in the cervical loop pulp tissues when compared to the body portions of the digested fresh dental pulp. Its mean percentage within the cervical loop area was 21% while its percentage within the body was only 4% (Figure 3-5).

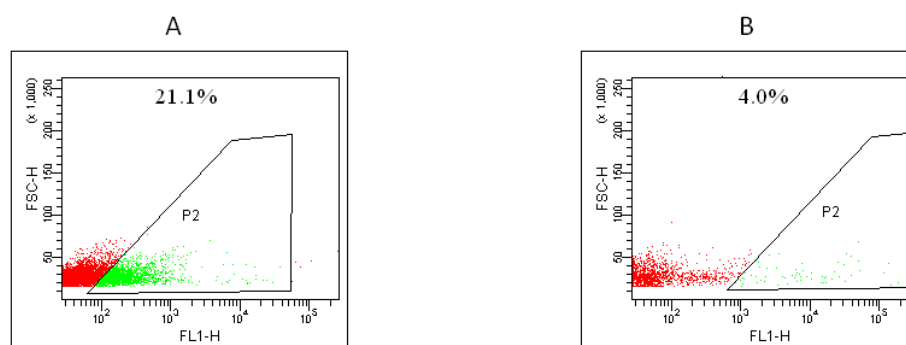


Figure 3-5: Flow cytometric analysis of Thy-1 expression in the digested cervical loop and the body pulp tissues of PN5 mouse incisor. Thy-1 mean expression in the cervical loop area pulp tissue was 21.1% (A) whereas in the body it was only 4.0 % (B). P2 represents dental pulp tissue cells that express positive levels of FITC. The controls included IgG2b FITC stained dental pulp cells.

3.2.3 Thy-1 expression in mouse incisor dental pulp tissues *in vivo*

3.2.3.1 Thy-1 expression in mouse incisor dental pulp tissues at different developmental stages

To examine Thy-1 expression in mouse incisor dental pulp tissues at different developmental stages, whole mount *in situ* hybridization was performed on PN1, PN5 and adult mouse mandibular incisors. At all three stages tested, Thy-1 expression was maintained within the cervical loop area although it varied in intensity. In postnatal day 1 (PN1) mice, Thy-1 expression was higher in the mesenchymal tissue close to the labial cervical loop. However, it was also expressed in the mesenchymal tissue close to the lingual cervical loop and the mesenchymal tissue between two cervical loops at a lower intensity (Figure 3-6, A, A'). In addition, Thy-1 had slightly weak expression associated with the blood vessels in the dental follicle and dental pulp (Black and white arrows in figure 3-6, (a) and (a') respectively). In PN5 mice, Thy-1 expression was similar to PN 1 stage but more intense (Figure 3-6, B). vibratome sections showed that it was expressed at the tip of the labial cervical loop and the mesenchymal tissue related to and in between the two lingual and labial cervical loops region (Figure 3-6, B''). Also, it had expression in the blood vessels of the dental follicle (Black arrow in figure 3-6, b) and dental pulp (White arrows in figure 3-6, b'). Thy-1 expression in adult mouse incisor dental pulp was still restricted to labial and lingual cervical loops area but was less intense (Figure 3-6, C and C'). Moreover, it had weak expression associated with the blood vessels in the dental follicle and the dental pulp (Black and white arrows in (c) and (c') respectively).

To conclude, Thy-1 expression, although it varied in intensity, was restricted to the cervical loop area in all three stages of mouse incisors investigated.

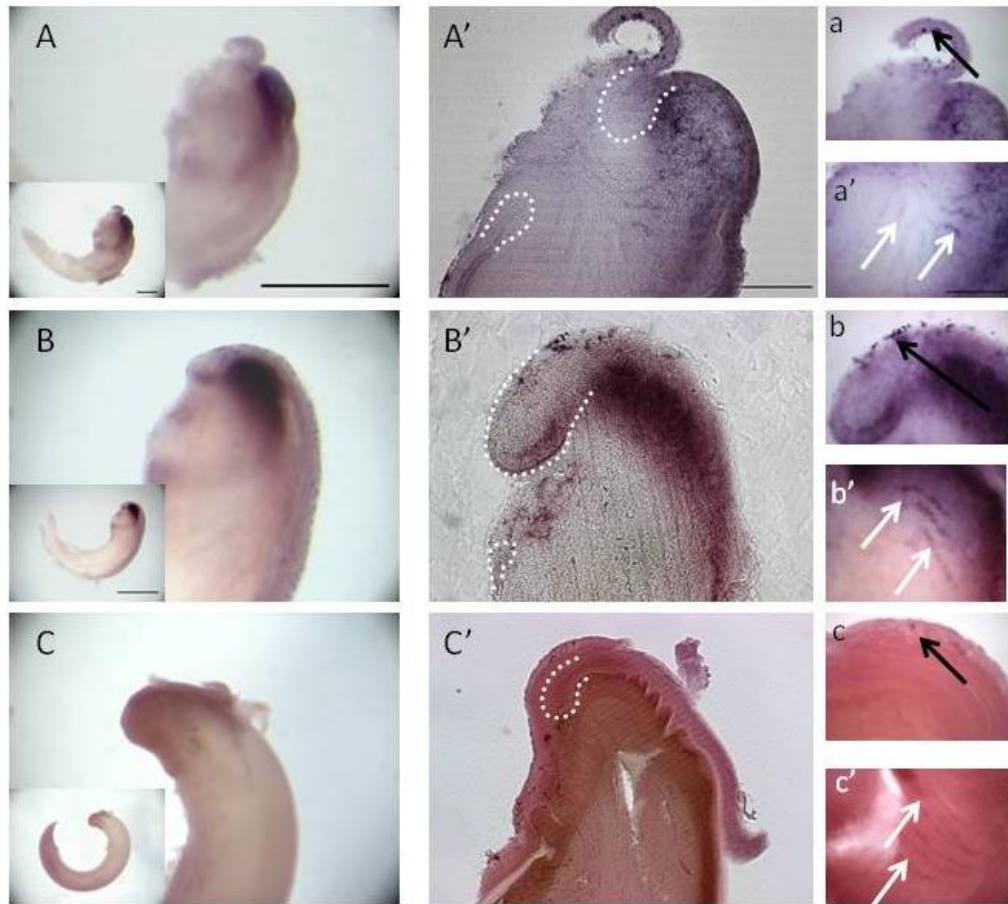


Figure 3-6: Thy-1 expression in mouse mandibular incisors. (A, A')Thy-1 expression in PN1 mouse mandibular incisor is intensely restricted to the mesenchyme close to the labial cervical loop. Also, it is slightly expressed in the mesenchymal tissue related to the lingual cervical loop and the mesenchymal tissue between two cervical loops (A). (a, a') vibratome sections show Thy-1 expression in the blood vessels of dental follicle (Black arrow) and dental pulp (White arrows). Whereas in PN5 mice, it is strongly expressed in the mesenchymal tissues close to the labial and lingual cervical loops, in addition to the mesenchymal tissue between them and tip of the labial cervical loop (B, B'). (b, b') Vibratome sections show Thy-1 weak expression correlated to the blood vessels of dental follicle (Black arrow) and dental pulp (White arrows). Thy-1 expression in adult mouse incisor still restricted to the cervical area but less intensified (C, C'). (c,c') Vibratome sections show Thy-1 weak expression related to blood vessels of dental follicle (Black arrow) and dental pulp (White arrow). White dotted lines represent the labial and lingual cervical loops. Scale bar =500μm in (A-C') and 250 μm in (a-c')

3.2.4 Thy-1 expression in mouse incisor dental pulp *in vitro*

3.2.4.1 Thy-1 expression decreases with increasing number of cell passages

The Thy-1⁺ population in PN5 mouse incisor dental pulp cell culture was investigated. Using flow cytometry, the proportion of Thy-1⁺ cells were quantified at four different time points; 1) digested dental pulp tissues, 2) initial explant culture (passage 0), 3) passage 2 and 4) passage 4. This experiment was repeated independently three times and the mean value was calculated. In culture, the Thy-1⁺ population percentage declined during culture expansion. In digested fresh pulp tissues, the percentage of Thy-1⁺ cells was 23.6%±1.2 (Figure 3-7, A), and this decreased to 17.8%±0.9 at initial explant culture (Figure 3-7, B). Then in passage 2, it declined again to 6.8%±0.8 (Figure 3-7, C) to finally reach 1.8%±0.1 in passage 4 (Figure 3-7, D). A summary of Thy-1⁺ cells percentages in mouse incisor dental pulp cells is shown in (Figure 3-8).

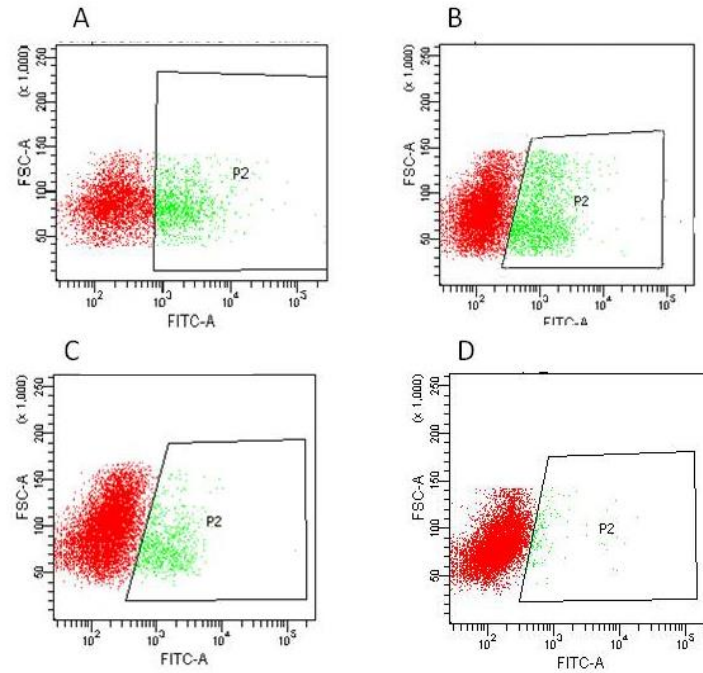


Figure 3-7: Thy-1⁺ cells in PN5 mouse whole dental pulp cells. Thy-1⁺ cells percentage in digested fresh pulp tissue (A) was 22% while in passage 0 (B) it went down to 18.8%. Again, it reduced to 7.8% in passage 2 (C). Finally, it went down to 1.7% in passage 4 (D). Number of gated cells in A is 5000, in B, C and D is 10,000. P2 represents cells that express positive levels of FITC. The control included IgG2b FITC stained cells. Results are representative of 3 independent experiments.

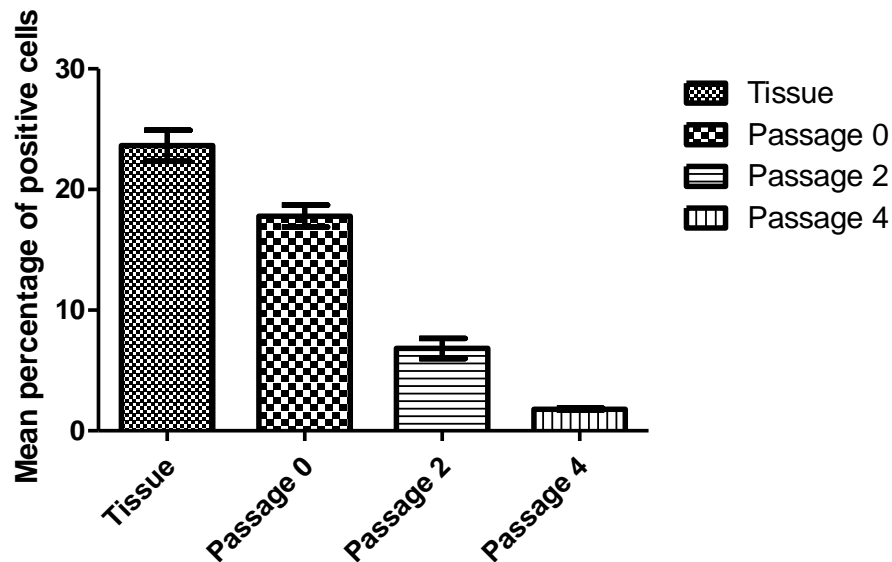


Figure 3-8: Summary of Thy-1⁺ populations in incisor dental pulp in culture. Mean value of Thy-1⁺ populations in each passage is presented. Statistical analysis using one-way ANOVA test revealed that Thy-1⁺ populations were significantly decreased in culture during consecutive passaging where P value (<0.0001). Bonferroni multiple comparison test showed significant difference between different groups. Error bar=SD. Results are representative of 3 independent experiments

3.2.4.2 Thy-1⁺ sorted cells do not maintain expression in cell culture

An investigation was carried out to discover whether sorted Thy-1⁺/Thy-1⁻ cells are able to maintain/gain Thy-1 expression in culture. Expanded PN5 mouse dental pulp cells were sorted using MACS to separate cells to Thy-1⁻ and Thy-1⁺ populations as previously described in (Chapter 2, 2.7). Unsorted cells, Thy-1⁺, Thy-1⁻ and Thy-1⁺/Thy-1⁻ in a ratio of 1:1 were plated in 12-well plates in triplicates. Cells were expanded in culture for only one passage to minimize any variance that might happen during culture. The percentage of Thy-1⁺ cells in these samples was analysed before and after expanding the cells using flow cytometry as described previously in (Chapter 2, 2.6). The experiment was repeated three times and the mean value calculated. The percentage of Thy-1⁺ cells significantly declined in all cell populations. In unsorted cells, the Thy-1⁺ population declined from 18.3% in passage 0 to 13.2% in passage 1. The Thy-1⁺ sorted population percentage decreased from 73.1 % in passage 0 to 24% in passage 1, while in mixed Thy-1⁺/Thy-1⁻ population, Thy-1⁺ cells percentage declined from 39% in passage 0 to 13% in passage 1.

To conclude, in all populations, Thy-1⁺ cells declined in culture, but this was more significant in sorted positive cell populations. This indicates that Thy-1⁺ sorted cells lose their expression of Thy-1 rapidly in culture. A summary of Thy-1 sorted and unsorted populations' percentages is shown in (Figure 3-9).

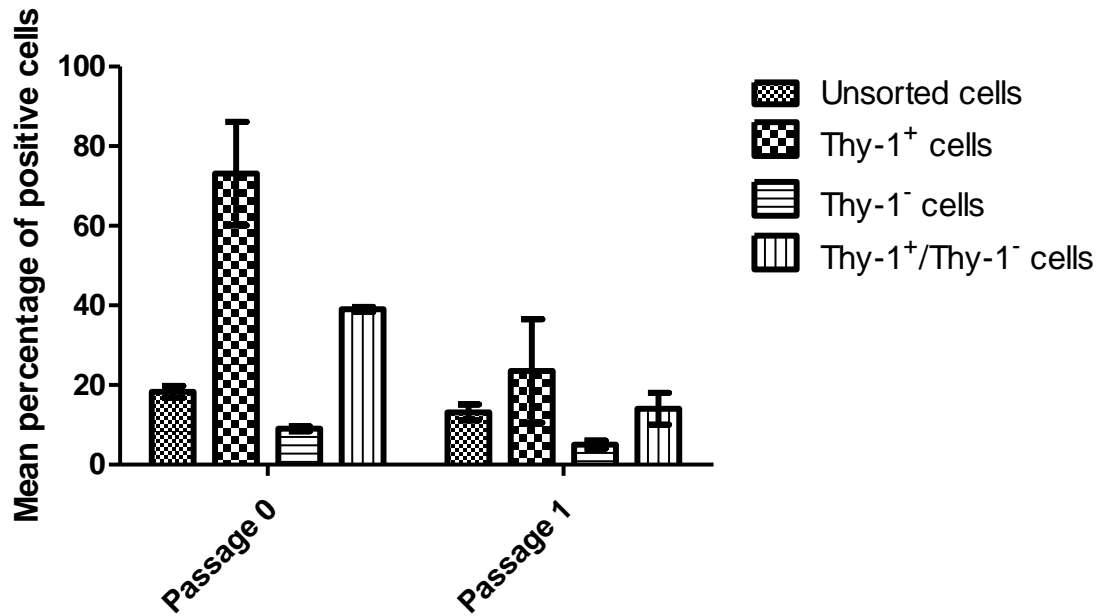


Figure 3-9: Thy-1 mean expression in sorted and unsorted cells. Thy-1⁺ cells percentage significantly decreased in unsorted cells from 18.3% to 13.2%, $p < 0.05$ ($p=0.015$). In Thy-1⁺ populations, there is a highly significant sharp declining from 73.1% to 24% $P < 0.05$ ($p=0.004$). Whereas in Thy-1^{+/−} population, Thy-1⁺ cells percentage decreased from 39% to 13% ($p=0.01$) and in Thy-1[−] population, the percentage went from 9% to 5% ($p=0.01$). Error bar =Mean \pm SD of triplicate samples. Results are representative of 3 independent experiments.

The morphology of cells can be important in many contexts. In culture, the morphology indicates the status of the cells, both in terms of the health and differentiation. Sorted Thy-1⁺ cell culture was monitored and compared with the sorted Thy-1⁻. In passage 1, after 5 days in culture, Thy-1⁺ cells were fewer in number compared to Thy-1⁻ cells which were almost confluent. Thy-1⁺ cells were heterogeneous; cells had round, fibroblastic, neural, flat and multi-angle appearance (Figure 3-10, A). Thy-1⁻ cells were more homogenous, cells were mostly round, refractile cells along with a layer of fibroblastic spindle shaped cells (Figure 3-10, B). In summary, Thy-1⁺ sorted cells were few in number and grow poorly in culture compared to Thy-1⁻ cells

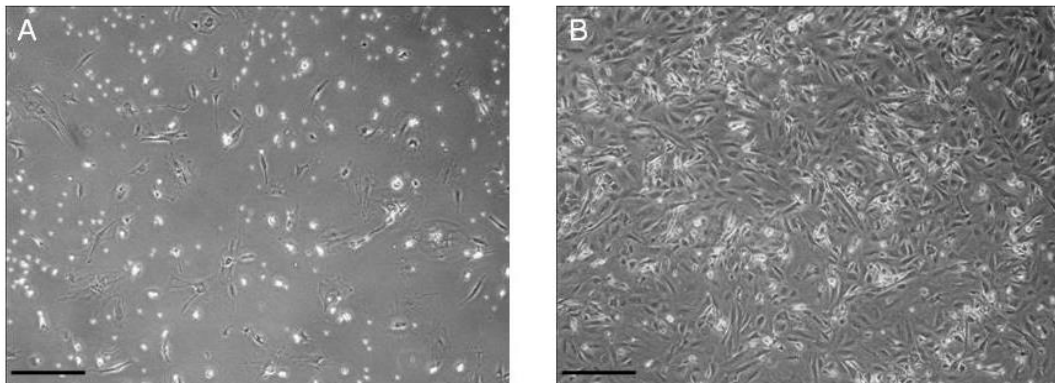


Figure 3-10: Morphology of Thy-1⁺ and Thy-1⁻ cells after 5 days in culture. Phase contrast micrograph showing that Thy-1⁺ cells (A) were fewer in number and hardly growing in culture when compared to the nearly confluent Thy-1⁻ cells (B) Scale bar = 200 μ m.

3.2.5 Characterization of mouse incisor dental pulp stem cells

3.2.5.1 In dental pulp cell culture

To further characterise mouse dental pulp stem cells, dual color flow cytometry with other mesenchymal stem cell markers was performed. CD146, a member of the immunoglobulin superfamily, has been used to prospectively isolate bone marrow and dental pulp MSC (Sorrentino et al., 2008, Shi and Gronthos, 2003). Using antibodies against CD146 and CD133 along with Thy-1, putative dental pulp stem cells were identified as subpopulations. Double staining of CD146 and Thy-1 revealed three different populations CD146⁺/Thy-1⁻, CD146⁺/Thy-1⁺ and CD146⁻/Thy-1⁺. These subpopulations were monitored in digested fresh dental pulp, passage 0 and 5.

In primary dental pulp tissue digests, the percentage of the CD146⁺/Thy-1⁻ population was 0.7%, increased to 3.2% in passage 0 and then declined to 0.6% in passage 5. The proportion of double-positive CD146⁺/Thy-1⁺ cells was 1.7% in pulp tissue digests, increased to 10.6% in passage 0 and in passage number 5, it declined to 0.3%. The CD146⁻/Thy-1⁺ population sharply declined from 21% in fresh pulp tissue digests to 1.9% in passage 0 then to 0.2% in passage 5 (Figure 3-11, A and B).

CD133, a transmembrane pentaspan protein initially described as a human hematopoietic stem cells (Yin et al., 1997), is used to isolate stem cells from numerous tissues such as bone marrow, brain, kidney, liver and dental pulp (Bussolati et al., 2005, d'Aquino et al., 2007a, Yin et al., 1997, Kordes et al., 2007, Lee et al., 2005). Dental pulp stem cells were also characterized based on double staining with CD133 and Thy-1. Dual staining with CD133 and Thy-1 revealed other three cell subpopulations in dental pulp. CD133⁺/Thy-1⁻, CD133⁺/Thy-1⁺ and CD133⁻/Thy-1⁺. In fresh pulp tissue, CD133⁺/Thy-1⁻ population was 9.7% reduced

to 2.6% in passage 0 and to 0.7% in passage 5. CD133⁺/Thy-1⁺ population was 3.5% in pulp tissue digests, increased to 7.5% in passage 0 but their percentage decreased to 0.2% in passage 5. CD133⁻/Thy-1⁺ population reduced from 19.6% in pulp tissue digest to 6.8% in passage 0 and to 0.2% in passage 5 (Figure 3-11, C and D). The presence of these subpopulations indicates the complexity of MSC niche inside the mouse incisor dental pulp.

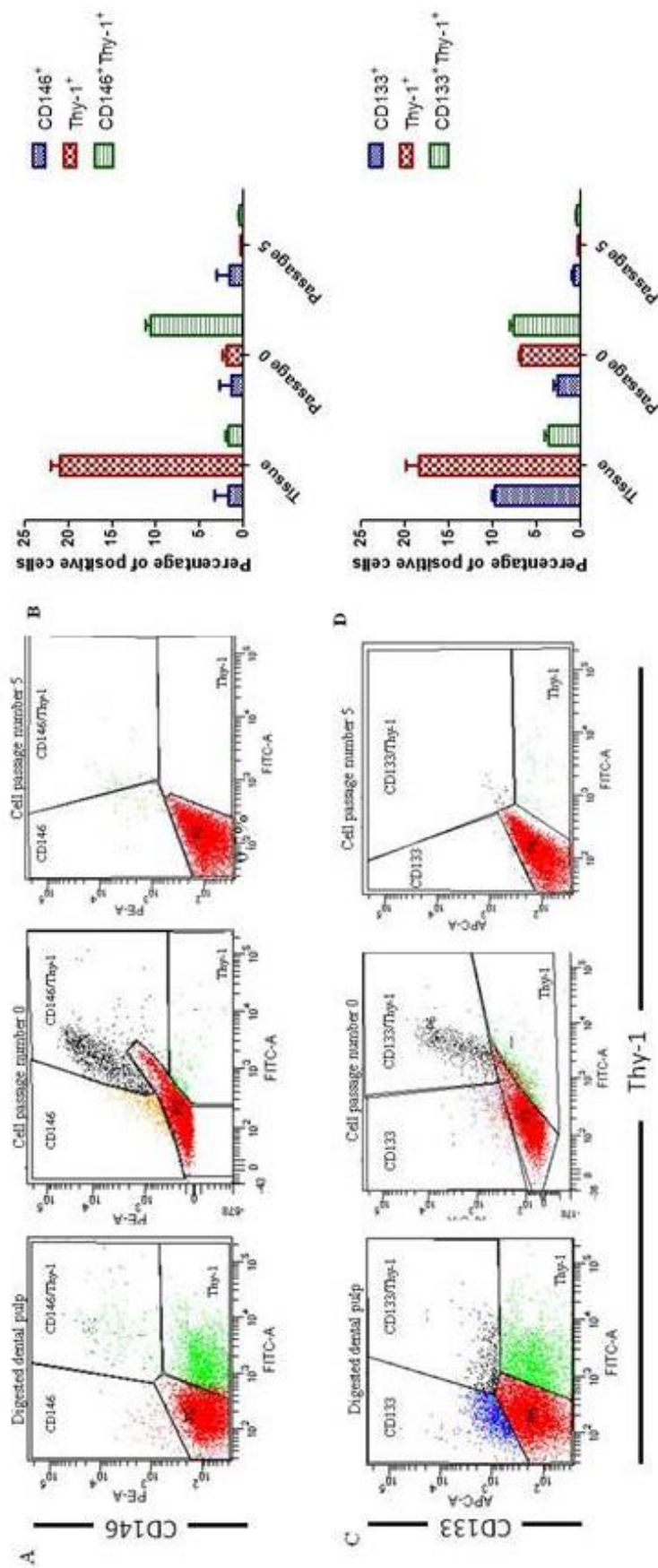


Figure 3-11: Characterization of dental pulp stem cells. Co-expression of Thy-1 with CD146 (A,B) and Thy-1 with CD133 (C,D) in whole PN5 mouse mandibular incisor dental pulp cells , passage 0 and passage 5. In A and C, Number of gated cells is 5000-10,000 cells in each reading. In B and D Error bar =Mean \pm SD. Results are representative of 3 independent experiments.

3.2.5.2 Attempting to locate Thy-1, CD146 and CD133 positive cells in dental pulp tissue using immunohistochemistry

Localization of CD133 and CD146 stem cell markers together with Thy-1 inside the dental pulp tissue will help to identify these putative mesenchymal stem cell subpopulations inside the mouse incisor dental pulp. Immunohistochemistry showed that CD146 staining was restricted to the perivascular tissues and endothelial cells (Figure 3-12, A and A'). Using published immunostaining methods for CD133 and Thy-1 antibodies, positive signals were hard to detect (Figure 3-12, B, B', C and C'). The control did not show any staining (Figure 3-12, D and D'). Collectively, the expression of the mesenchymal stem cell marker CD146 was associated with perivascular and vascular areas while CD133 and Thy-1 expression were hard to locate in the mouse incisor pulp tissue.

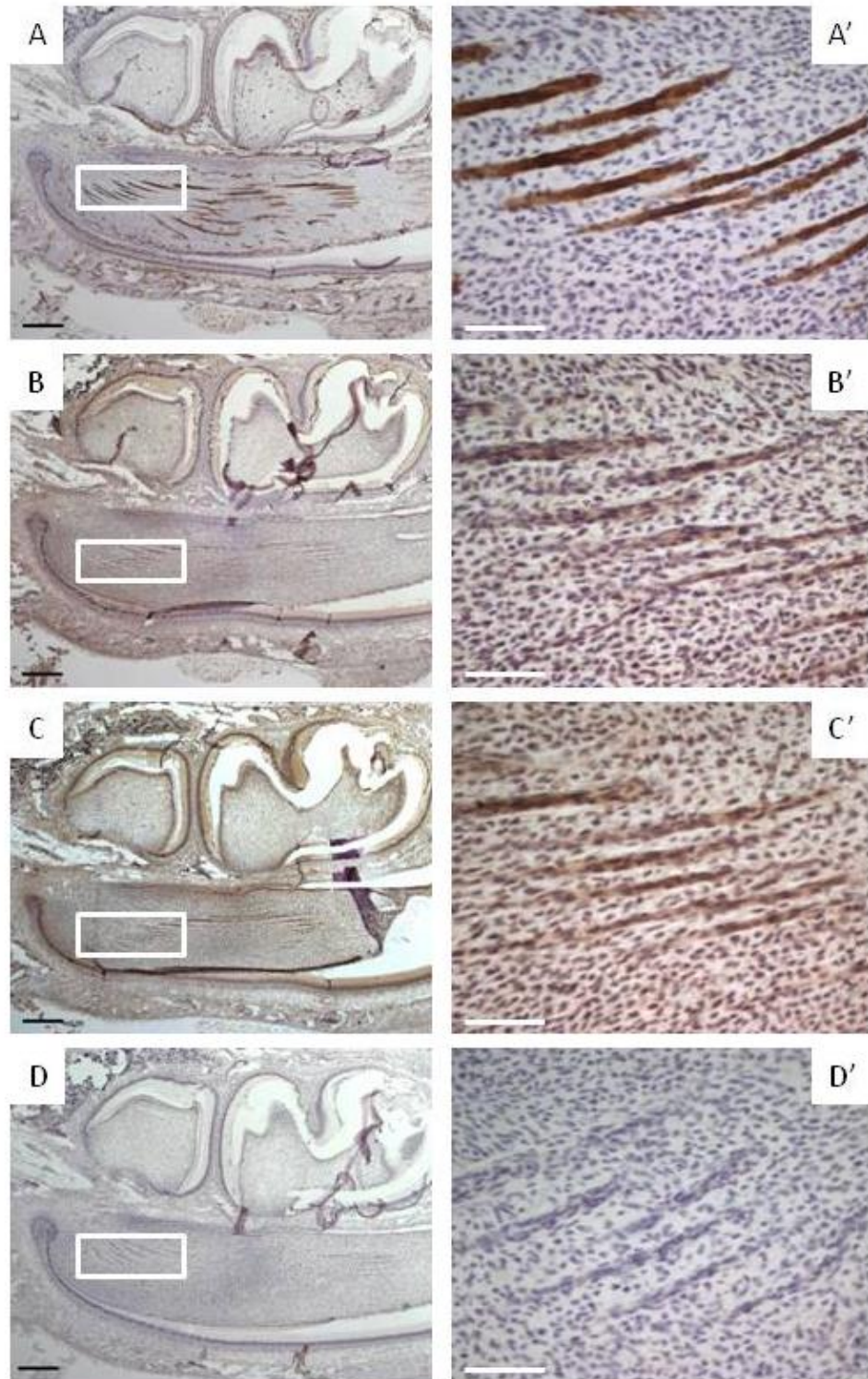


Figure 3-12: Immunodetection of CD146, Thy-1 and CD133 in PN5 mouse mandibular incisor dental pulp. CD146 expression is limited to the blood vessels and perivascular cells in the pulp tissue and the dental follicle (A, A'). CD133 and Thy-1 positive signals were hard to be detected (B, B', C and C'). The control was counterstained with haematoxylin (D, D'). Scale bar=500 μ m (A-D) and 250 μ m (A'-D'). These figures are representative of 3 independent experiments.

3.2.6 Osteogenic differentiation of incisor dental pulp stem cells

To examine the differentiation potential of mouse incisor dental pulp stem cells, osteogenic differentiation was induced as described in (Chapter 2, 2.17) at passage 2 and 4 using commercial osteogenic media or normal α MEM media as a control. In both passages, mineralization was detected three days after the addition of mineralization-inducing media and increased thereafter. At day 21, the induction was terminated. In passage 2, the entire culture dish was covered by a mineralized tissue layer, while in passage 4, there was patchy distribution of mineralized nodules. Interestingly, the percentage of the Thy-1⁺ population was investigated prior to each osteogenic induction. At passage 1, the Thy-1⁺ population was 17.5% whereas at passage 3, it was 4.5% which may suggest a relationship between the level of Thy-1 expression and the osteogenic potential of mouse incisor dental pulp cells (Figure 3-13).

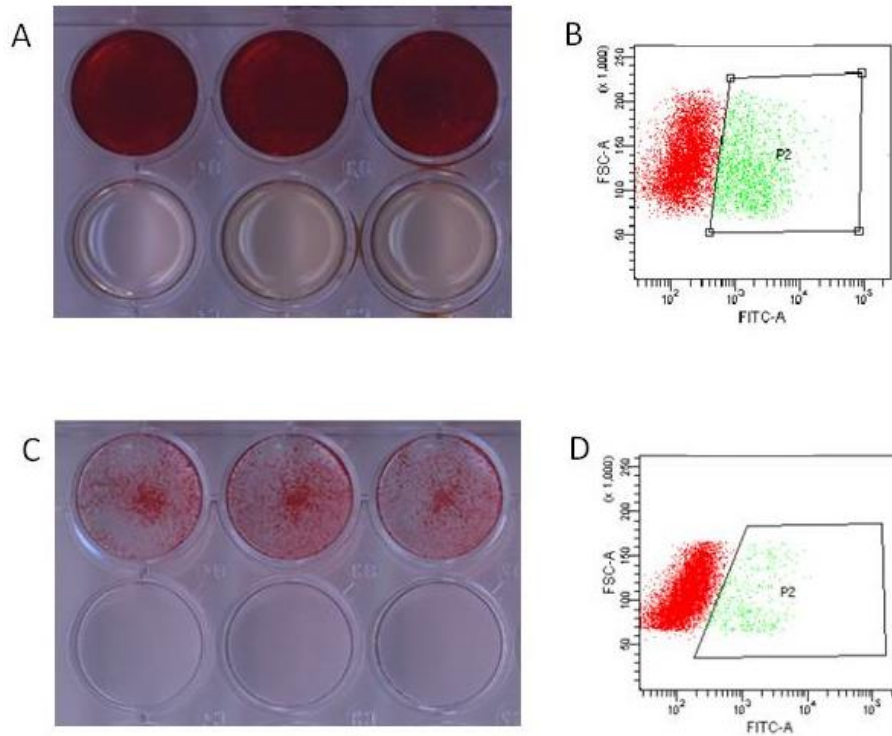


Figure 3-13: Osteodifferentiation of dental pulp stem cells. Osteodifferentiation of dental pulp stem cells at passage number 2 (A) was detected by Alizarin red staining. Thy-1⁺ population percentage was 17.6% (B). At passage number 4 (C) dental pulp stem cells osteodifferentiation was much weaker as they formed patches of calcified tissue and the Thy-1⁺ population was 4.5% (D). In both passages, the control did not show any signs of calcification. P2 represents dental pulp cells that express positive levels of FITC. The controls included IgG2b FITC stained dental pulp cells. Results are representative of 3 independent experiments.

3.3 Discussion

3.3.1 Stem cells within mouse incisor dental pulp

The mouse incisor tooth is an excellent model to analyze certain aspects of stem cell regulation and function. It erupts continuously throughout the animal's life due to the presence of stem cells in the cervical loop area (Harada et al., 1999). Epithelial stem cells are located mainly in the labial cervical loop (Harada et al., 1999) while the mesenchymal stem cells are located in the perivascular and periapical area of the incisal dental pulp (Feng et al., 2011). MSCs represent a mixture of different cell types. One of several issues that have hampered the widespread use of MSCs for regenerative dentistry is the lack of surface markers specifically identifying MSCs from their differentiated progeny (Kaltz et al., 2010). The development of microarray methods for large-scale analysis of mRNA gene expression make it possible to search systematically for key molecules (Duggan et al., 1999).

A previous stem cell microarray was performed in our lab (Mantesso and Sharpe, unpublished, 2008). This microarray compared stem cell gene expression between mouse incisor and molar dental pulp tissues. Four genes which were up-regulated in the incisor dental pulp tissues were selected. Their selection was based on their expression during tooth development. They are: Cellular Retinoid Acid Binding Protein-1 (Crabp-1), Hydroxy Urea Sensitive-1 (Hus-1), Zinc Finger protein, subfamily 1A, 1 (Znfn1a1) and Thy-1. Thy-1 has already been described in detail in (Chapter1, Section1-6), and none of the others have previously been reported to be expressed in mouse developing incisors except Crabp1 (<http://bite-it.helsinki.fi>).

Crabp-1 is one of two highly homologous but different Crabp-1 genes (Crabp-1 and Crabp-II). Crabps are small intracellular proteins that bind with high affinity to

Retinoic Acid (RA) which is a naturally occurring metabolite of vitamin A and plays a vital role in the normal physiology of vertebrates (Norris et al., 1994). In the continuously growing incisor of adult rats, Crabp-1 was present throughout the pre-secretory and secretory ameloblasts. However, it disappeared from that layer during its maturation phase. In the adjacent dental mesenchyme of the developing pulp Crabp-1 was expressed especially in the layer immediately beneath the fully differentiated odontoblasts. In contrast, Crabp-1 expression was not prominent in the odontoblast layer itself. This expression suggests a role for this molecule during differentiation and hard tissue genesis (Berkovitz et al., 1993). Nevertheless, a direct role of Crabps during tooth development is unlikely as Ch55, a synthetic RA analogue that does not bind to Crabps, had the same effects as RA on *in vitro* incisor development (Bloch-Zupan et al., 1994). Moreover, Crabp1 homozygous mutant mice are normal without any malformations indicating that Crabp1 does not play a crucial role in the RA signalling pathway (Gorry et al., 1994).

Hus-1 is one member of the 9-1-1 complex, a cell cycle checkpoint pathway. Checkpoint proteins halt cell cycle progression and promote repair or apoptosis in response to DNA damage, thereby preventing mutation accumulation and suppressing tumor development (Weiss et al., 2003). Mouse Hus1 (mHus-1) was found to be expressed in a variety of adult tissues such as heart, mammary glands, liver and testes. Also, it was found to be expressed at several stages of embryonic development in addition to its expression in embryonic stem cells. Taken together, these data indicate that *mHus-1* is expressed in a pattern consistent with its presumed role as a component of a cell cycle checkpoint protein complex that surveys for damaged DNA (Weiss et al., 1999). Hus1 inactivation in the mammary epithelium resulted in genome damage that induced apoptosis and led to depletion of *Hus1*-null

cells from the mammary gland, a process which is hampered by *p53*-deficiency (Yazinski et al., 2009). Hus-1 has not been previously reported to be expressed in any dental tissue.

Znfn1a1 also known as Ikaros, is a hematopoietic-specific member of a family of zinc finger transcription factors that is essential for specification in lymphoid lineages (Nichogiannopoulou et al., 1999). Ikaros mRNA is not only restricted to sites of both adult and embryonic lymphopoiesis, but it was also found to be an essential factor for the development of all lymphoid lineages (Molnar and Georgopoulos, 1994). During foetal development, it is required at the earliest stages of T-cell and B-cell specification. However, in the adult, lymphoid lineages rely on *Ikaros* at distinct phases of their development (Georgopoulos et al., 1997). It has been reported to function as a tumour suppressor in the T-cell lineage (Winandy et al., 1995). Similar to Hus-1, Ikaros has not previously been reported to be expressed within any dental tissues.

To localize the expression of these genes in the incisor pulp tissues, whole mount *in situ* hybridization was performed. Interestingly, all of them were restricted to the cervical loop area where mesenchymal stem cells are hypothesized and found to be located (Feng et al., 2011, Seidel et al., 2010a). This may suggest that they are expressed by mesenchymal stem cells located in this area.

To analyze and compare the gene expression in the body and cervical loop portions of the dental pulp, Affymetrix GeneChip microarrays were carried out. Within the mouse incisor dental pulp, there were genes up-regulated in the cervical loop area compared to their expression in the body of the incisor. The results of this microarray were compared to those of the stem cell one as the lists of up-regulated

genes in both microarrays were compared together. Surprisingly, only three genes were in common. The first gene was Distal-less homeobox 5 (Dlx5) which encodes a member of a homeobox transcription factor gene family similar to the *Drosophila* distal-less gene (Sajan et al., 2011). The encoded protein may play a role in bone development and fracture healing. Dlx5 is expressed in all stages of tooth development, suggesting a possible role for this gene in tooth formation. Its asymmetric expression in the dental epithelium and dental mesenchyme during tooth development may contribute to the complex patterning of human tooth shape (Davideau et al., 1999). Dlx5 was up regulated in both the molar and the cervical loop area of the pulp tissue.

The second gene was Thrombospondin 1 (Thbs-1) a matricellular glycoprotein first discovered in activated platelets (Lawler et al., 1978). TSP-1 is the best-studied member of the thrombospondin (Tsp) family, which consists of five extracellular calcium-binding multifunctional proteins. Tsp-1 and Tsp-2 are structurally similar, and are expressed on the cell surface during physiological events. A variety of normal cells, including endothelial cells, fibroblasts, adipocytes, smooth muscle cells, monocytes, macrophages, and transformed cells such as malignant glioma cells, secrete Tsp-1 (Wight et al., 1985). Tsp-1 binds to protein components of the extracellular matrix, such as fibronectin. In this way, Tsp-1 is stored in the extracellular matrix where it folds and changes its conformation. Tsp-1-specific domains bind to proteoglycans, membrane proteins such as integrins, and other matrix proteins expressed by a variety of cells (Bornstein, 1995). Tsp-1 was up regulated in the molar and incisor body pulp tissues.

The third gene was Thy-1 a 25–35-kDa glycosylphosphatidylinositol-binding glycoprotein expressed on human bone marrow and cord blood CD34⁺ haematopoietic progenitor cells (Craig et al., 1993). Previously, Thy-1 was widely used as a marker for mouse haematopoietic stem cells (Petersen et al., 1998) and human haematopoietic progenitor cells (Craig et al., 1993). *Thy-1* is used as a general mesenchymal stem cell marker (Horwitz et al., 2005). *Thy-1* is also expressed in keratinocyte stem/progenitor cells (Nakamura et al., 2006). In addition, *Thy-1* is expressed in human dental pulp cell lines (Suguro et al., 2008). Recently, Thy-1 was reported to be expressed in the subodontoblastic zone which is thought to be a source of newly differentiated odontoblasts after pulp trauma (Hosoya et al., 2012).

In comparing the two microarrays, *Thy-1* expression was up-regulated in both the incisor and cervical loop pulp tissues. Due to its up- regulation in the cervical loop area which harbours the incisor mesenchymal stem cells in addition to its usage as a stem cell marker in other tissues, *Thy-1* was selected for further analysis to detect its expression and identify more mesenchymal stem cell niches within the mouse incisor pulp tissues.

3.3.2 Thy-1 expression in mouse incisor dental pulp

Thy-1 expression in the mouse incisor dental pulp was investigated using *in situ* hybridization at different postnatal developmental stages; PN0, PN5 and adult stage. It was revealed that in all stages, Thy-1 expression was limited to the mesenchymal part of the cervical loop area where the mesenchymal stem cells of mouse incisor dental pulp are located (Feng et al., 2011). In addition to its expression in the apical part of the pulp tissues, it also had slightly weak expression in the vascular area of the incisor dental pulp and the follicular blood vessels where mesenchymal stem cell

niches are believed to exist *in vivo* (Feng et al., 2011, Shi and Gronthos, 2003). Collectively, these data suggest that Thy-1 expressing cells in mouse dental pulp are located in sites famous for being mesenchymal stem cells niches inside the mouse incisor dental pulp tissues.

(Balic and Mina, 2010), reported that Thy-1 expression in mouse incisor dental pulp tissues from ages of PN18-21 was less than those from ages PN5-7. In in situ studies in our study, Thy-1 expression in adult incisors was less in intensity and more limited in area when compared to PN0 and PN5 expression. Therefore, it might be argued that Thy-1 expression started to decrease with aging as the MSCs number decrease with age (Stolzing, et al. 2008). In early passages of hBMSCs, Thy-1 was one of the stem cell markers that decreased significantly with age although it was expressed in all samples regardless the donor age (Stolzing, et al. 2008). A similar decrease in the MSC numbers as a function of age has also been documented in satellite cells (Gibson and Schultz, 1983). Nevertheless, further analysis to confirm reduced Thy-1 expression in adult tissues is required.

Although Thy-1 mRNA had been detected in PN5 mouse incisor dental pulp, its protein detection failed. Further immunohistochemical staining tests are needed to locate its protein in mouse dental pulp tissues.

In *ex vivo* manipulation studies, MSCs are known to undergo phenotypic changes, losing expression of some markers while also acquiring new ones (Jones et al., 2002). Due to specific culture conditions and the duration prior to individual passages, some MSCs may change *in vitro* (Dazzi et al., 2006). In cell culture studies in this project, the Thy-1⁺ population in digested dental pulp tissues was 23.6%. With successive passaging, this population declined to reach 1.9% in passage

4. This agrees with the study in which Thy-1⁺ population declined in PN5-PN7 old murine molar pulp cells from 95% down to 35% after 7 days in culture (Balic et al., 2010). Similarly, the Thy-1⁺ population decreased in neonatal normal human epidermal keratinocytes to reach 2.6%±0.6 by passage 10. Nevertheless, the Thy-1⁺ population did not decline when cultured with a feeder layer (Nakamura et al., 2006). However, the Thy-1⁺ population in murine bone marrow increased during the cultivation period to reach 44% in passage 3 (Eslaminejad et al., 2007). This difference in the pattern of Thy-1⁺ expression between mouse dental pulp and mouse bone marrow populations could be due to different tissue properties, as bone marrow is more cellular when compared to the dental pulp (Shi et al., 2001).

Incisor dental pulp cells were expanded in culture and sorted at passage 0 for of two reasons. First, it was the highest level of Thy-1⁺ expression in culture. Second, it was necessary to expand the cells to get enough cell number for cell sorting. Sorted cells were grown for only one passage to avoid any great modification in cell phenotype or gene expression. In human HSC sorted cells, the Thy-1⁻ population generated Thy-1⁺ cells *in vitro* and *in vivo* (Notta et al., 2011). In contrast, in this project, the Thy-1⁺ population in sorted and unsorted cells declined in culture, similar to whole dental pulp culture. Nevertheless, there was a difference between these cell populations. In sorted Thy-1⁺ cell populations, the mean percentage showed a highly significant decline between passage 0 and 1, compared to other populations.

MSCs reside in tissues in specific niches, which encompass all of the elements surrounding the stem cells when they are in their immature state, including the non-stem cells, ECM and soluble molecules found in that locale, all of these act together to keep the stem cells in their undifferentiated state. The loss of any of these

elements could be a signal for stem cell differentiation to occur for regenerating or repopulating a tissue (reviewed in (Kolf et al., 2007a). Accordingly, the significant decrease in Thy-1⁺ population mean percentage in the Thy-1⁺sorted population could be due to cells trying to find a balance between Thy-1⁺ and Thy-1⁻populations by losing Thy-1 expression. Alternatively, the Thy-1⁺ population may rely on Thy-1⁻ cells to maintain expression. This could be confirmed by the finding that mean Thy-1 level in both the unsorted and Thy-1^{+/-} mixed populations are equal, probably because both of them have higher percentage of Thy-1⁻ population compared to the Thy-1⁺ sorted one. Thy-1⁺ cells grew poorly in culture when compared to the Thy-1⁻ cells which increases the probability of their need to maintain contact with Thy-1⁻ cells in order to keep their growth and hence their expression.

To conclude, maintaining the Thy-1⁺ population in culture is difficult as they lose their Thy-1 expression with successive passaging. Further studies to grow this population in different conditions are required to confirm that. Also, growth rate curve for Thy-1⁺and Thy-1⁻ cells is needed to compare their growth for longer in culture.

3.3.3 Characterisation of dental pulp stem cells

The precise identity of DPSCs remains a challenge because of the lack of a single specific stem cell marker. The most common method of cell purification to enrich the population of a specific cell type is by labelling cell lineages with fluorescent antibodies and then purifying them by FACS. This again requires the identification of specific cell surface markers for particular cell types (Mitsiadis et al., 2011). CD146, also known as S-Endo 1-associated antigen, MelCAM or MUC18, is a transmembrane glycoprotein that is constitutively expressed in the whole human endothelium, irrespective of its anatomical site or vessel calibre (Lehmann et al.,

1989). However, CD146 expression is not only restricted to Endothelial Cells (ECs); it has also been observed on several other cell types, including melanoma cells, smooth muscle cells and follicular dendritic cells (Lehmann et al., 1989). CD146 with other markers such as Neural/Glial Antigen 2 (NG2) and Platelet-derived growth factor receptor-beta (PDGFR β) have been used to locate pericytes –a cell type believed to be a MSCs- in a wide range of human tissues including the dental pulp mesenchyme (Crisan et al., 2008b). Overall, CD146 is considered one of the MSC markers that is present on endothelial and smooth muscle cells (Shi and Gronthos, 2003). Therefore, CD146 was selected to characterize the dental pulp stem cells alongside Thy-1.

CD146⁺ and CD146⁺/Thy-1⁺ populations behave similarly in culture. CD146 and Thy-1 are expressed by pericyte (da Silva Meirelles et al., 2008). The CD146⁺/Thy-1⁺ population probably represents the pericyte population inside the dental pulp. However, further investigation is needed to confirm this by using other pericyte markers such as NG2 and PDGFR β . The CD146⁺ population detected by flow cytometry and localized by immunohistochemistry could represent the endothelial cells inside the dental pulp. Double staining with an endothelial cell marker such as CD31 would be necessary to locate them precisely in the dental pulp. Also, more *in vitro* analysis is required to determine the nature and potentiality of each population.

CD133 (prominin-1) was the first in a class of novel pentaspan membrane proteins to be identified in both humans and mice (Mizrak et al., 2008). Mouse prominin-1 was described as a novel marker of neuroepithelial progenitor cells (Weigmann et al., 1997), whereas its human ortholog is a novel antigen—defined by the widely used Monoclonal Antibody (MAb) AC133, with its expression limited to haematopoietic stem and progenitor cells (Yin et al., 1997). However, using another antibody that

recognized human CD133 independently of glycosylation, CD133 was found to be expressed in several tissues including adult kidney, mammary gland, trachea, salivary gland, placenta, pancreas, digestive tract and testes, similar to mouse prominin (Florek et al., 2005, Weigmann et al., 1997). Due to its expression by haematopoietic progenitors, interest has been directed towards the potential of CD133 as a cell surface marker of adult stem cells. Human circulating CD133 cells were induced to undergo either endothelial or cardiomyocytic differentiation *in vitro* (Bonanno et al., 2007). Bussolati *et al* (2005) showed that a population of CD133 cells isolated from the adult human kidney were capable of both self-renewal and multi-lineage differentiation *in vitro* and *in vivo*, and could contribute to renal tissue regeneration by differentiation to form both epithelial and endothelial lineages (Bussolati et al., 2005). In addition to the haematopoietic system, CD133 is also expressed on endothelial progenitor cells (EPCs), which play a role in angiogenesis and neo-vasculogenesis during both tumour growth and wound healing (Ribatti, 2004). In this project, A CD133⁺ population inside the dental pulp may represent EPCs which is necessary for blood vessel formation in the developing dental pulp of PN5 old mice (Balic and Mina, 2010). However, detection of CD133 positive signals in wax sections of PN5 mouse incisor dental pulp failed despite using different published immune-staining protocols. Using cryo-sections is recommended for further study to locate CD133 in mouse incisor dental pulp. Also, CD133 is a promising stem cell marker in several other tissues such as human and mouse brains and mouse embryonic stem cells (Kania et al., 2005). In human dental pulp cells, the double positive Thy-1⁺ CD133⁺ subpopulation was considered as a source of undifferentiated potentially multipotent cells (d'Aquino et al., 2007b). Accordingly,

in this project, the CD133⁺ Thy-1⁺ double positive population could represent a separate subpopulation of stem cells.

All together these data suggest that Thy-1⁺ population is heterogeneous. Since Thy-1 is expressed by a wide variety of cells (Craig et al., 1993, Saalbach et al., 1999), it is not an exclusive marker for mesenchymal stem cells inside the mouse incisor dental pulp. Probably using other MSC markers together with Thy-1 will reveal more subpopulations.

3.3.4 Osteogenic/odontogenic differentiation of dental pulp stem cells

Murine DPSCs exhibit a multipotent character since they are capable of differentiating into various cell types including adipocytes, chondrocytes and osteoblasts (Waddington et al., 2009, Grottkau et al., 2010, Balic and Mina, 2010). However, (Pittenger et al., 1999) reported that, some of the clonal derived populations were able to differentiate into all three lineages, but other clonal populations were lacking differentiation into at least one lineage. Notably, all clonal populations were able to undergo osteogenesis. After long-term culture together with successive passaging, the rate of differentiation declined (Muraglia, et al. 2000), although the potential to differentiate into adipogenic and osteogenic progeny did not change (Müller-Sieburg and Deryugina 1995). In porcine bone marrow, MSCs with more than 15 passages expressed decreased levels of the bone morphogenetic protein (*BMP-7*) and reduced activity of alkaline phosphatase and consequently reduced potentiality toward osteogenic differentiation (Vacanti et al., 2005).

In rat dental pulp, Thy-1⁺ cells located to the subodontoblastic zone were able to form bone both *in vivo* and *in vitro*. Moreover, histomorphometric analysis demonstrated that the bone-like tissue area formed by the cells of the Thy-1^{high}

fraction was markedly larger than those of the Thy-1^{low} fraction (Hosoya et al., 2012). Also, in foetal rat limb bone, Thy-1 was suggested as a marker for osteoprogenitor cells (Nakamura, et al. 2010). In this project, mouse dental pulp stem cells were able to form bone in two different cell passages with different percentages of Thy-1⁺ population. Bone formation was more evident in the earliest passage with a higher percentage of Thy-1⁺ population which may suggest a relationship between the potentiality toward bone formation and Thy-1 level which further suggests Thy-1 expression as an indicator of osteogenic potentiality. This could be further confirmed by comparing the osteogenic differentiation between Thy-1⁺ cells and Thy-1⁻ cells. However, from previous experiments, it was shown that not only did the Thy-1⁺ percentage decrease with passaging, but so also did the percentages of other MSC markers such as CD146 and CD133. Thus, a decrease in osteogenic potential could also be due to losing MSCs in culture probably as a result of their differentiation or oxidative damage accumulation (Ebert, et al. 2006) which induce senescence (Byun, et al. 2005; Finkel and Holbrook 2000) and inhibit osteoblastic differentiation (Mody, et al. 2001). Further analysis is needed to explain decreased osteogenic potentiality, ex. monitoring proliferation, indices of cellular aging as reactive oxygen species (ROS) and lipofusion. In addition, adipogenesis and chondrogenesis of these cells should be tested to investigate if only osteogenesis was affected with the previous mentioned factor

Chapter 4 : Lineage Tracing of Thy-1 Expressing Cells in Mouse Dental Pulp

4.1 Introduction

MSCs within their tissue of origin *in vivo* are not yet known, because of the lack of a specific marker which allows their unambiguous identification (Bianco et al., 2008). However, cell lineage tracing experiments, that are now increasingly feasible with modern technologies and detections systems, will shed light on these unresolved issues about the *in vivo* nature of the MSCs in their intact tissues (Augello et al., 2010). In lineage tracing, a single cell is marked in such a way that the mark is transmitted to the cells progeny, resulting in a set of labelled clones (Kretzschmar and Watt, 2012). This method can thus definitively identify adult stem cells (Haegebarth and Clevers, 2009, Joyner and Zervas, 2006).

The regulatory elements of the murine Thy1.2 gene were used to drive Cre recombinase expression in the nervous system (NS) of transgenic mice. Thy1-Cre lines exhibited transgene expression in several regions of the central and peripheral nervous systems, including the cerebral cortex, cerebellum, spinal cord, retina, and dorsal root ganglion (Campsall et al., 2002).

To study major therapeutic strategy for Alzheimer's disease (AD) patients, Thy-1 Cre mice were used to selectively delete Presenilin-1 (PS1) expression in mouse brain (Dewachter et al., 2002).

In this study, to trace Thy-1 expressing cells inside the mouse incisor dental pulp, Thy-1Cre transgenic mice, where Cre is driven by Thy-1 promoter, were mated with one of the reporter mouse lines, Rosa26R (R26R). In Thy-1Cre;R26R transgenic

mice where Cre is expressed, the loxP-flanked STOP sequence is deleted and these cells and all their descendants are permanently marked by expression of lacZ (Soriano, 1999). How and where Thy-1 cells are maintained in mouse dental pulp tissue in vivo is not known. Thus, Thy-1 expressing cells in mouse dental pulp were traced during embryonic and postnatal stages using Thy-1Cre;R26R transgenic mice.

4.2 Results

4.2.1 Validation of Cre Expression

To confirm effective recombination between Cre and loxP sites, and prove that Cre expression was driven by Thy-1 gene promoter, whole mount *in situ* hybridization for Cre and Thy-1 was performed. Cre had similar expression to Thy-1 (previously described in our results); both of them were expressed in the cervical loop area. In PN5 mouse incisors, they were expressed next to the labial side. However, the Thy-1 expression area was slightly wider than Cre. Also, they were expressed in tissues between the labial and lingual sides with very weak expression close to the lingual side (Figure 4-1, A and B).

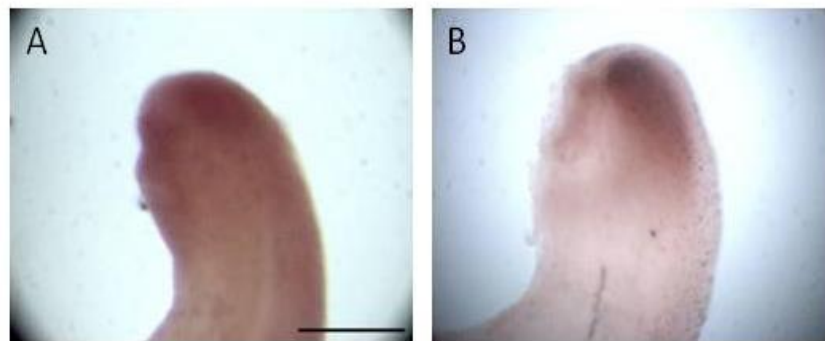


Figure 4-1 Cre and Thy-1 co-localization in PN5 mouse mandibular incisor: (A) Cre and Thy-1 (B) were expressed in the cervical loop area close to the labial side and the apical tissues between the two cervical loops. Thy-1 expression was more extended on the labial side of the cervical loop area. Scale bar in A and B = 500µm. Results are representative of 3 independent experiments.

This shows that recombination of Cre with LoxP sites was successful and Cre was activated only in Thy-1 expressing cells. Nearly all cells expressing, or that expressed Thy-1 before, would be blue in color.

4.2.2 Thy-1 lineage tracing in mouse developing tooth germ during pre-natal stage

4.2.2.1 Thy-1 Cre; R26R LacZ⁺ cells at E14.5

4.2.2.2 In E14.5 developing incisors, few LacZ⁺ (Thy-1⁺) cells were present condensed ectomesenchymal cells of the developing tooth dental papilla (White arrow in figure 4-3, A). In the developing molar, more LacZ⁺ cells were present in the dental papilla when compared to the incisor (White arrow in

4.2.2.3 **Figure 4-2: Frontal cryosection of E14.5 mouse embryo mandible showing LacZ⁺ cells in Thy-1Cre^{+/-};R26R^{+/-} developing tooth germ., B).** The control samples, Thy-1 Cre^{+/+}; R26R^{+/+} did not show any LacZ staining (

Figure 4-2: Frontal cryosection of E14.5 mouse embryo mandible showing LacZ⁺ cells in Thy-1Cre^{+/-};R26R^{+/-} developing tooth germ., C and D). This finding shows that Thy-1⁺ cells contribution to dental papilla (that will form the pulp tissue) formation during tooth development, is more restricted in incisors than in molars at this stage of development.

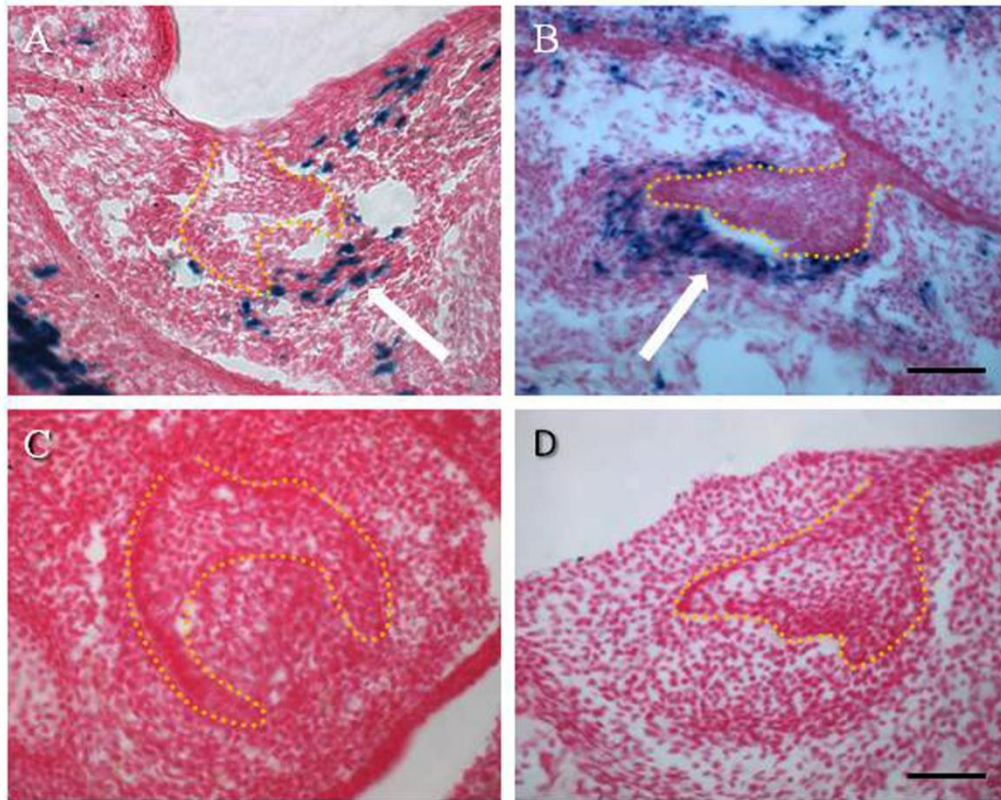


Figure 4-2: Frontal cryosection of E14.5 mouse embryo mandible showing LacZ^+ cells in $\text{Thy-1Cre}^{+/-};\text{R26R}^{+/-}$ developing tooth germ. (A) In the developing incisor, few LacZ^+ cells were present in the dental papilla. While in the developing molar (B) more LacZ^+ cells were contributing to the formation of dental papilla. $\text{Thy-1 Cre}^{+/+};\text{R26R}^{-/+}$ incisor (C) and molar (D) controls did not show any LacZ^+ cells. Scale bar=250 μm . Results are representative of 3 independent samples.

4.2.2.4 At E17.5, LacZ⁺ cells were related to future odontoblasts

At E17.5, the mouse tooth germ has already acquired the bell shape stage, and dark stained cells of the dental papilla are aligned opposite the light-stained dentino-enamel junction (Tecles et al.). These cells represent the future odontoblasts which will secrete dentin (Kerley, 1975). Although LacZ⁺ cell number was more in molars when compared to incisors, in both LacZ⁺ cells were found throughout the dental papilla. Few of these cells were aligned opposite the DEJ (White arrows in Figure 4-4, A and D) as future odontoblasts and ameloblasts precursor cells (Black arrows in figure 4-4, A and D). Also, very few LacZ⁺ cells were associated with the developing blood vessels in the dental papilla (Black arrows in figure 4-4, B and E). The control samples did not show any LacZ⁺ staining (Figure 4-4, C and F). These findings show that Thy-1 contribution to the future dental pulp was still restricted at this stage. However, Thy-1⁺ cells were presented by a few tooth-forming cells including future odontoblasts, and very few were related to the developing blood vessels walls, a mesenchymal stem cell niche previously described in the literature (Shi and Gronthos, 2003, Feng et al., 2011).

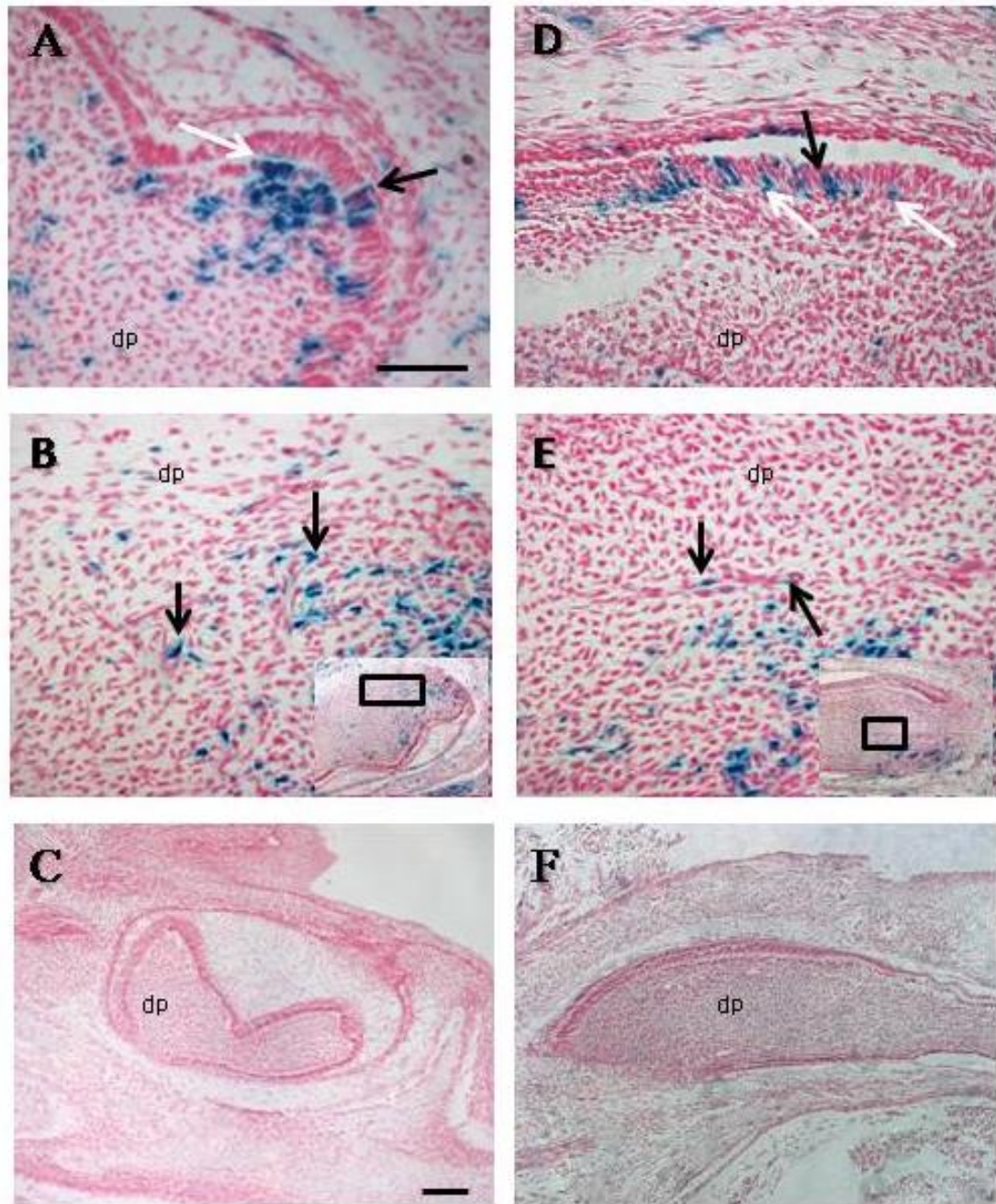


Figure 4-3: Longitudinal section of E17.5 mouse mandible showing LacZ⁺ cells in Thy-1Cre^{+/-};R26R^{+/-} mouse developing tooth germ. A and D Molar and incisor tooth germ respectively show LacZ⁺ cells' contribution to odontoblasts precursors cells (White arrows), ameloblasts precursors (Black arrows). (B) and (E) Higher magnification of the black boxed area inset shows very few LacZ⁺ cells in molar and incisor dental papilla related to blood vessels walls (Black arrows). Thy-1Cre^{+/-}; R26R^{+/+} Molar (C) and incisor (F) did not show any Lac-Z⁺ cells. dp=dental pulp. Scale bar = 250μm in A, B, D and E, 500μm in C, E and F. Results are representative of 3 independent samples

4.2.3 Thy-1 lineage tracing in mouse tooth dental pulp during post-natal stage.

To further explore the contribution of LacZ⁺ cells in Thy-1Cre^{+/-};R26R^{+/-} mouse tooth dental pulp formation, LacZ staining was investigated during the post-natal stages, at PN5 and PN60.

4.2.3.1 In PN5, LacZ⁺ odontoblast, blood vessels and pulp cells contributed to dental pulp formation

At PN5, a larger number of LacZ stained cells contributed to dental pulp tissue formation of molars and incisors when compared with the pre-natal stages (Figure 4-5, A and E). Labelled cells were mostly found in patches rather than individual cells. At PN5, both molar and incisor dental pulps contained LacZ⁺ cells in the subodontoblastic zones as well as LacZ⁺ odontoblasts with their processes extending into the dentin (Black arrows in figure 4-5, B and F). While most of them were distributed throughout the pulp tissue, some of them were always linked to the blood vessels inside the dental pulp tissue (White arrows in figure 4-5, C and G). Although a large number of LacZ⁺ cells contributed to dental pulp tissue formation in molars and incisors, these cells formed approximately 30% of dental pulp cells. The presence of positive and negative populations indicates that another population of LacZ⁻ cells (Thy-1⁻) also contribute to dental pulp formation together with LacZ⁺ cells (Thy-1⁺). Control samples did not show any positive LacZ staining (Figure 4-5, D and H). These results show that although LacZ⁺ cells increased in number in post-natal mouse teeth, they formed part, but not all, of the dental pulp cells including odontoblasts. Some of these LacZ⁺ cells were linked to blood vessels walls and sub-odontoblastic zones, which are stem cell niches inside the dental pulp.

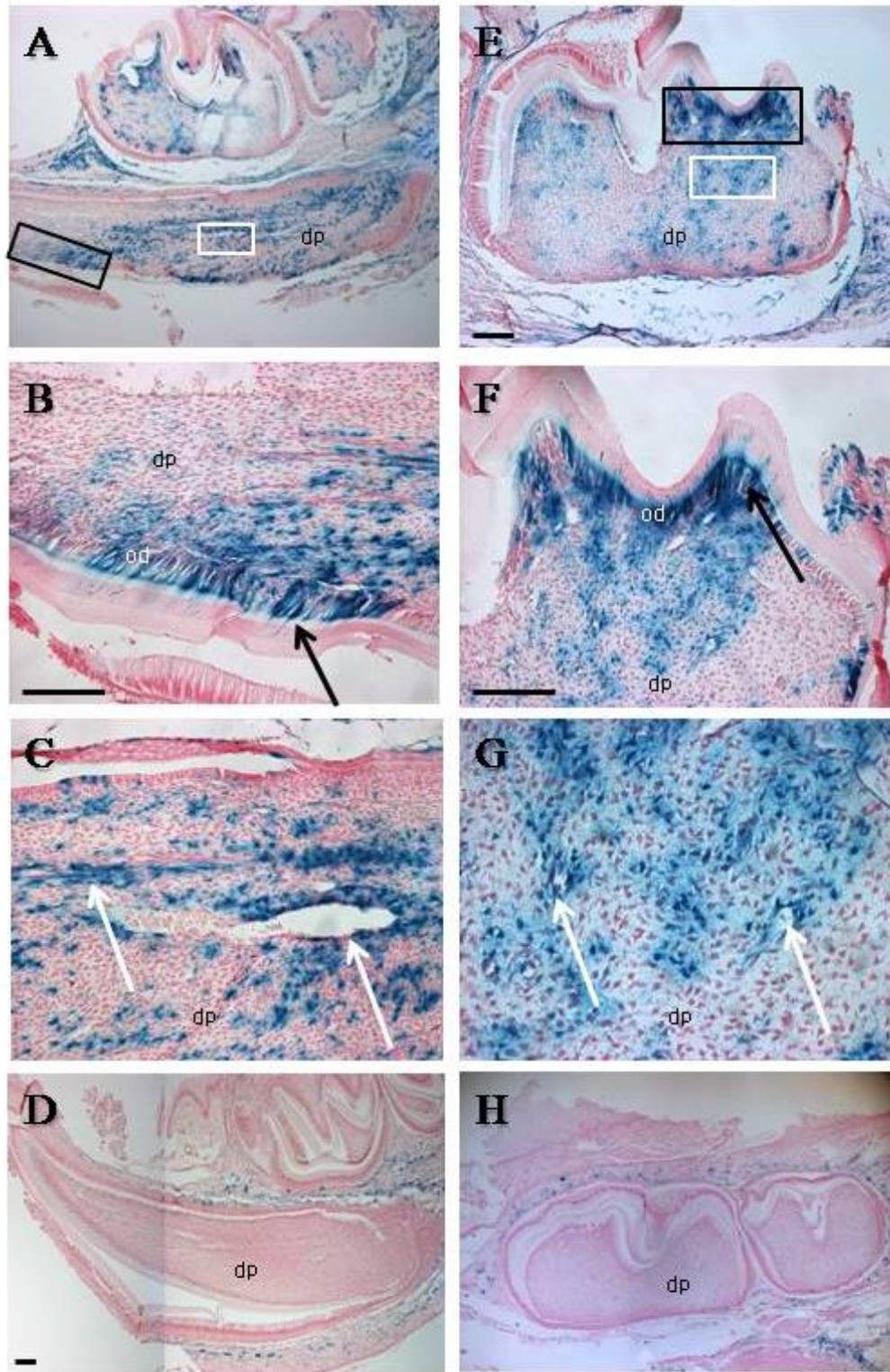


Figure 4-4: Longitudinal sections of PN5 Thy-1Cre^{+/-};R26R^{+/-} mouse mandibular molar and incisor showing LacZ⁺ cells in their dental pulp tissue. A and E show LacZ⁺ cells distribution throughout the molar and incisor dental pulp respectively. LacZ⁺ cells contributed to sub-odontoblastic zone and odontoblasts in molars and incisors in order (B and F). Some of LacZ⁺ cells are related and linked to the blood vessels inside the dental pulp in incisors (C) and molars (G). Thy-1 Cre^{+/-}; R26R^{+/-} incisors (D) and molars (H) control teeth did not show any LacZ staining in their pulp tissues. dp=dental pulp, od=odontoblast. Scale bar= 250µm in (C and G), and 500 µm in (A, B, D, E, F and H). Results are representative of 3 independent samples.

Interestingly, LacZ⁺ odontoblasts (Approximately 25 cells in incisor Longitudinal section (L.S)) in PN5 Thy-1Cre^{+/-};R26R^{+/-} incisor dental pulp were located in almost equal size bands alternated with LacZ⁻ ones. In addition to the presence of another stem cell population, this would suggest rhythmic odontoblast formation between LacZ⁺ and LacZ⁻ cells (White boxed area in figure 4-6, A, B, B' and B''). This was confirmed by cutting cross sections through the incisor to ensure that alternative bands formed as a result of rhythmic formation rather than wrong sectioning angulations. In cross sections, LacZ⁺ odontoblasts bands (Approximately 6 cells in incisor Transverse Section (T.S)) were still alternated with LacZ⁻ odontoblasts almost on the entire labial surface area of the incisor (Black boxed area in figure 4-6, C, and black arrows in C' and C''). This finding supports the hypothesis of rhythmic formation between LacZ⁺ (Approximately 150 cells in a patch) and LacZ⁻ odontoblasts, and suggests a complex system of MSC's interaction in the dental pulp.

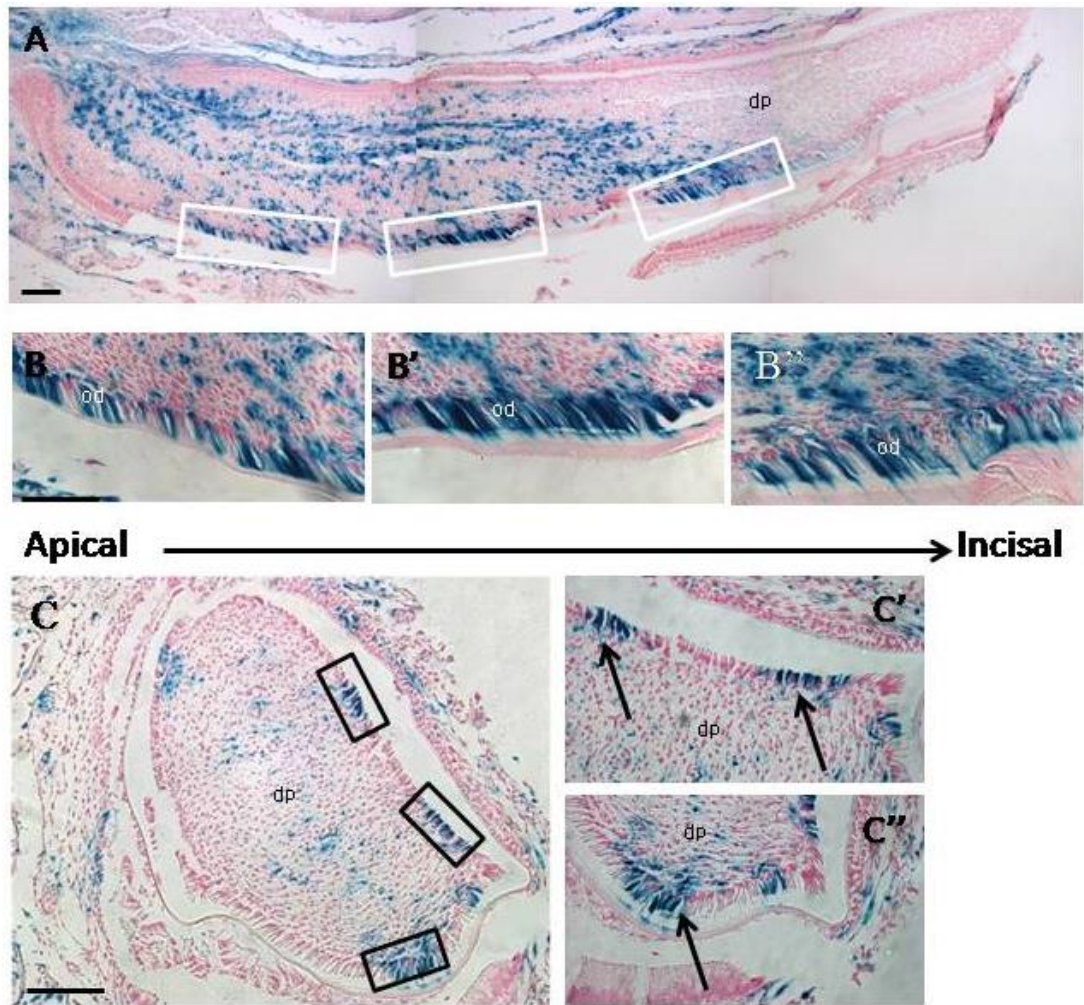


Figure 4-5: Rhythmic formation of LacZ⁺ odontoblasts in PN5 Thy-1Cre^{+/-};R26R^{+/-} mouse mandibular incisor. (A) Longitudinal section (L.S.) of Thy-1Cre^{+/-};R26R^{+/-} mouse incisor showing LacZ⁺ odontoblasts bands (White boxed areas). (B,B' and B'') higher magnification of white boxed area in A from apical to incisal direction respectively.(C) Transverse section (T.S.) of Thy-1Cre^{+/-};R26R^{+/-} mouse mandibular incisor showing alternated bands of LacZ⁺ odontoblasts (Black boxed areas). Higher magnification of the black boxed area in C' and C'' showing LacZ⁺ odontoblasts bands (Black arrows).dp=dental pulp, od=odontoblast. Scale bar= 500µm in (A and C), and 250 µm in (B, B',B'',C'andC''). Results are representative of 3 independent samples.

4.2.3.2 In PN60, LacZ⁺ odontoblasts, blood vessels and pulp cells still contribute to dental pulp formation and regeneration.

As mouse incisors are continuously growing to compensate for the incisor attrition resulted from eating, stem/progenitor cells are expected to proliferate to overcome this shortage. So, LacZ⁺ cells were expected to increase in number with age. In Thy-1 Cre^{+/-};R26R^{+/-} adult mouse (60 days old), LacZ⁺ cells formed almost all of the dental pulp tissue of the molar (Figure 4-7, E) including odontoblasts (Black arrow in figure 4-7, F) and also were linked to blood vessels (White arrow in figure 4-7, G). However, in Thy-1 Cre^{+/-};R26R^{+/-} adult incisors, LacZ⁺ cells contributed to part of the incisor dental pulp (Figure 4-7, A). Thy-1 expression was still linked mostly in odontoblasts (Black arrow in figure 4-7, B) and dental pulp blood vessels (White arrow in figure 4-7, C). Thy-1Cre^{+/+};R26R^{-/+} incisor and molar did not show any LacZ staining (Figure 4-7, D and H respectively).

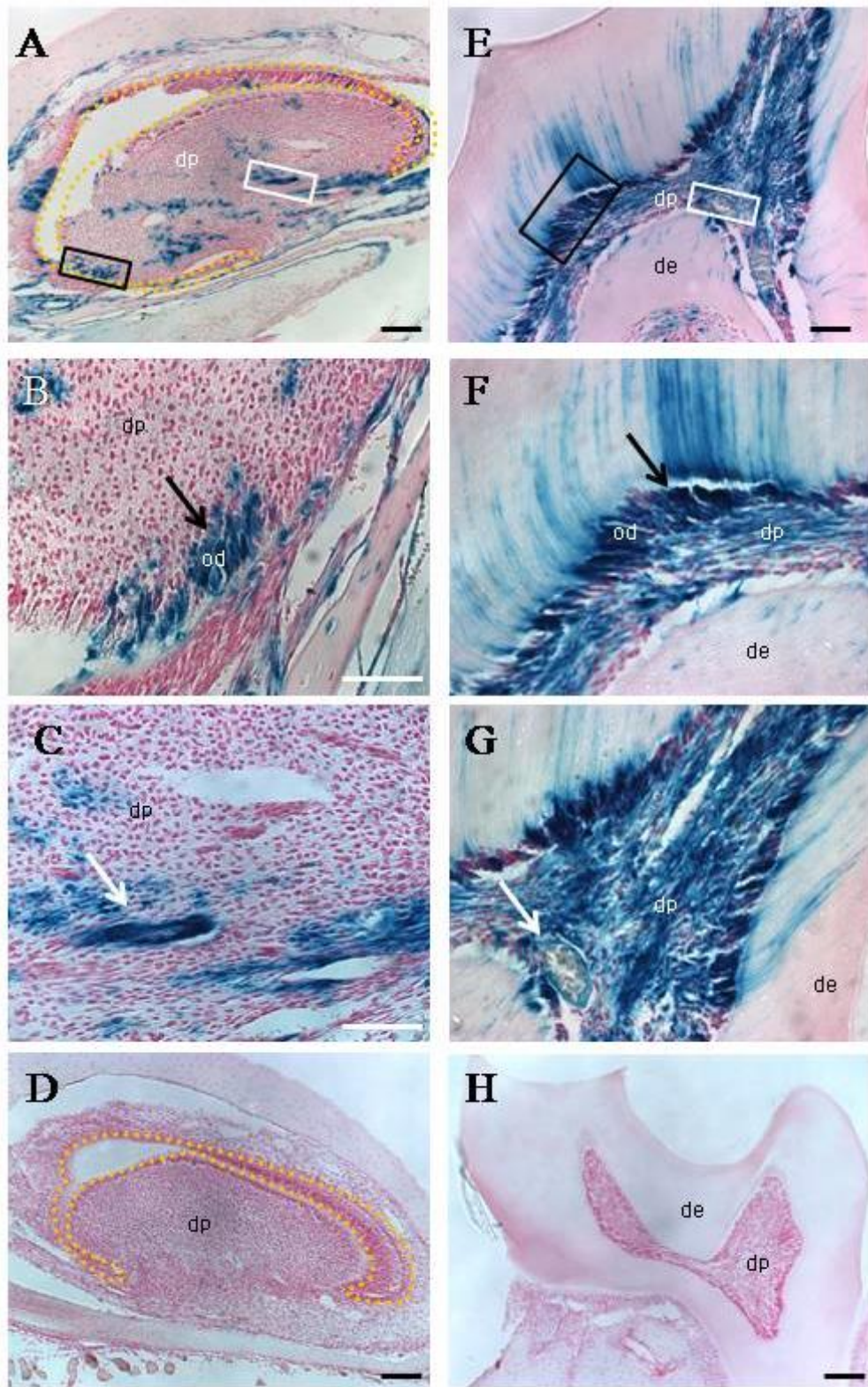


Figure 4-6: Longitudinal sections of PN60 Thy-1Cre^{+/-};R26R^{+/-} mouse mandibular incisor and molar. (A and E) LacZ⁺ cells in the cervical part of the mouse incisor and molar respectively. (B and F) Higher magnification of the black boxed area in (A) and (E) in order to show LacZ⁺ odontoblasts (Black arrow). (C and G) Higher magnification of white boxed area in (A) and (E) in order to show the correlation of LacZ⁺ cells with the blood vessels inside the dental pulp (White arrow). Thy-1 Cre^{+/-};R26R^{+/-} incisor (D) and molar (H) showed no LacZ staining. dp=dental pulp, od=odontoblast,

de=dentin. Scale bar = 500 μ m in (A, D, E and H) and 250 μ m in (B, C, F and G). Results are representative of 3 independent samples.

In adult mouse incisors, continuous attrition of the incisal tip leads to continuous regeneration and eruption of the incisor. In adult Thy-1 Cre^{+/+};R26R^{+/+} mouse incisor dental pulp, all cells including LacZ⁺ cells contributed to dental pulp formation in earlier stages are lost due to incisor continuous growth. So, LacZ⁺ cells traced in mouse adult incisor dental pulp are presumably derived from the mesenchymal stem cell niche at the apical end of the incisor. These LacZ⁺ cells were mainly in odontoblasts (Black arrow in figure 4-8, a) and linked to blood vessels (White arrows in figure 4-8,a'). However, not only LacZ⁺ cells, but also LacZ⁻ cells contributed to the dental pulp and odontoblasts at that stage. This suggests a complex mesenchymal stem cell niche.

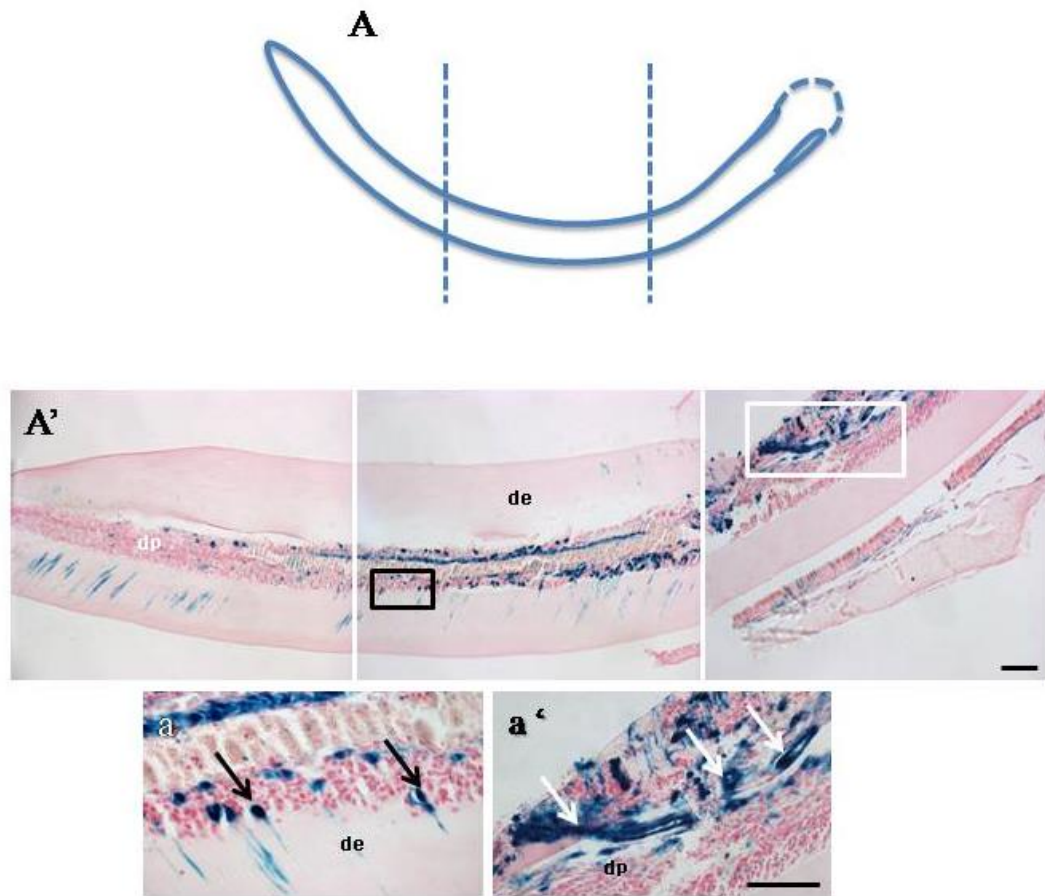


Figure 4-7: LacZ⁺ cells in Thy-1Cre^{+/-};R26R^{+/-} mouse mandibular incisor. (A) a diagram shows the continuously growing adult mouse incisors mesio-distal thirds. (A') shows the incisal third of an adult incisor. (a) Higher magnification black boxed area in (B) shows LacZ⁺ odontoblasts (Black arrows). (a') Higher magnification of white boxed area in (B) shows LacZ⁺ cells in blood vessels wall (White arrows). de=dentin, dp=dental pulp. Scale bar in (A')=500µm, in (a) and (a')=250 µm. Results are representative of 3 independent samples.

4.3 Discussion

While the multipotential capacity of MSCs has been proven *in vitro* (Pittenger et al., 1999), their *in vivo* identities are less understood. This is due to lack of specific markers for their identification (Bianco et al., 2008). Lineage tracing, a technique originally developed to study early embryos, represents by far the most powerful and reliable tool for identifying stem cells and for deciphering other aspects of tissue behaviour (Kretzschmar and Watt, 2012). Genetic lineage tracing is usually performed using the Cre-LoxP system. Thy-1 is one of the mesenchymal stem cell markers (Horwitz, et al. 2005) but its expression inside the mouse teeth dental pulp has not been investigated so far.

In Thy-1Cre;R26R mice, *Cre* recombinase is expressed under the control of the Thy-1 promoter. When crossed with R26R reporter mouse line, where the reporter gene is flanked by a floxed stop codon, cells expressing both constructs and their descendants are permanently stamped by the blue color of X-gal (Soriano, 1999). From our results, the efficacy of Cre recombination was confirmed by *in situ* hybridization. So, every LacZ⁺ cell found is expressing, or had expressed Thy-1 during its lifetime.

4.3.1 Thy-1Cre; R26R LacZ⁺ cells in early tooth formation

During tooth development, Cranial Neural Crest (CNC) derived ectomesenchyme contributes to the condensed dental ectomesenchyme during the bud stage and subsequently to the formation of the dental papilla and surrounding dental follicle (Chai et al., 2000). Accordingly, odontoblast, dentine matrix, most pulpal tissues and DPSCs were suggested to be derived from CNC (d'Aquino et al., 2009).

In E14.5 Thy-1Cre^{-/+};R26R^{-/+} mice, LacZ⁺ cells were present in the condensed ectomesenchymal tissue of both molars and incisors which suggests their neural crest origin. Sasaki et al. 2008, have demonstrated that adult rat DPSCs contain primitive stem cell subpopulations of neural crest origin, including Nestin⁺ precursor cells, Tuj1⁺ neuron cells, and S100⁺ glial cells (Sasaki, et al. 2008). These stem cells could generate neurospheres under serum-free culture conditions. Another study has shown that DPSCs express several neural crest-related markers such as S-100, Nestin, CD57, CD271 and Glial fibrillary acidic protein (reviewed in Yan, et al. 2011). By employing RT-PCR, immunofluorescent and flow cytometric analyses, it has been shown that subpopulations of SHED cells co-express neural crest cell markers such as p75NTR and SOX10 with MSCs markers such as CD146, indicating that adult dental MSCs retain the expression of neural crest cell genes (Mohamed, 2010). Staining of LacZ⁺ cells with neural crest cell markers such as Slug, Snail, and Sox10 in our mutants will confirm their neural crest origin. At E17.5 stage, polarized dental papilla cells (future odontoblasts) were LacZ⁺ revealing that these cells are derived from Thy1⁺ MSCs that contribute to the dental pulp formation.

Thy-1 expression in early stages of tooth development is in contrast with a recent study in which immunodetection in rat dental pulp failed to locate Thy-1 during the early stage of tooth development (Hosoya, et al. 2012). It could be argued that Thy-1 is membrane glycoprotein that displays species-specific in its pattern of expression (Tokugawa, et al. 1997). Although Thy-1 is expressed on thymocytes and splenocytes in mice, it is only expressed on thymocytes in rats. So, Thy-1 is not necessarily to be expressed in the same tissues at the same time in mice and rats, importantly, LacZ activity is activated in Thy-1 expressing cells or their progeny

even after they lose this expression (Soriano 1999), therefore these LacZ⁺ cells probably were expressing Thy-1 at a stage earlier to E14.5.

At E17.5 Thy-1Cre^{+/-};R26R^{+/-} tooth dental pulp, very few LacZ⁺ cells were close to or related to the blood vessel walls, which is the anatomical site of pericytes (Crisan et al., 2008b). This may suggest them as the pericytes of the dental pulp which form one of the mesenchymal stem cell niches inside the mouse incisor pulp (Shi and Gronthos, 2003, Feng et al., 2011). In a pericyte reporter mouse line (XLacZ4) during tooth development, some pericytes were clearly visible as signal cells at a close distance to the vessel walls as they entered the dental mesenchyme. Staining of these LacZ⁺ cells with one of the pericyte markers such as NG2 or α SMA will reveals their nature.

In summary, the presence of LacZ⁺ cells in the condensed ectomesenchymal tissue of the dental papilla indicates their neural crest origin. In addition, their association with blood vessels -although limited- identifies them as perivascular cells inside the dental pulp. These findings, in addition to the presence of LacZ⁺ polarized dental papilla cells (odontoblast precursors), suggest Thy-1⁺ cell contribution to dental pulp formation. Nevertheless, this contribution is limited.

4.3.2 Dental pulp stem cells *in vivo*

Many studies have shown that DPSCs play a role in dentin-pulp tissue regeneration (Gronthos et al., 2000b, Miura et al., 2003, Yu et al., 2007b). Although many single-factors can induce the differentiation of DPSCs, such as transforming growth factor β 1 (TGF β 1) alone or in combination with fibroblast growth factor 2 (FGF2) (He et al., 2008), the *in vivo* microenvironment of DPSCs is complex involving matrix components, mineral ions, and growth factors. Thus, the odontoblastic

differentiation induced by only one or two growth factors may cause undesirable biological changes in differentiated DPSCs (Yan et al., 2011). *In vivo*, human DPSCs recombined with hydroxyapatite-tricalcium phosphate (HA-TCP) ceramic powder, were able to generate typical dentin structures surrounded by odontoblast-like cells with long cytoplasmic processes when transplanted subcutaneously into immunocompromised mice (Gronthos et al., 2000a). Similar dentin formation could be detected *in vivo* when SHEDs were recombined with HA-TCP scaffolds (Miura et al., 2003, Batouli et al., 2003). Furthermore, DPSCs can produce dentin pulp-like complex in the scaffold and reparative dentin-like structure on the surface of dentin slices (Batouli et al., 2003, El-Backly et al., 2008). LacZ⁺ cells increased from the early stages of tooth development until adulthood in Thy-1 Cre^{+/-};R26R^{+/-} molars and incisors. Some of these cells were related to blood vessel walls and contributed to an increasing percentage of different cell types in the pulp tissue, including odontoblasts that were identified according to their anatomical position and histological characteristics.

Taken together, these characteristics suggest that, during mouse tooth development Thy-1⁺ progeny contribute to dental pulp and odontoblasts formation. As a result of the incisor continuous growth, cells including lacZ⁺ cells contributed in early tooth formation are lost. Accordingly, Thy-1⁺ cells traced in adult incisor are derived from the mesenchymal stem cell niche located at the cervical area of the incisor (Feng, et al., 2011). Thy-1⁺ cells in adult incisors showed a capacity for multilineage differentiation forming the complex and terminally differentiated odontoblast. However, alternation between LacZ⁺/LacZ⁻ odontoblasts zones and LacZ⁻ pulp cells indicates that another stem cell population(s) that is LacZ⁻ also contributed to dental pulp formation. These findings corroborate to results presented previously by Feng

et al., (2011), that by using genetic lineage tracing combined to response to injury analysis demonstrated that there is a pericyte-derived mesenchymal contribution to odontoblast formation, but it does not account for all cells differentiation. Taken together, it indicates a heterogeneous nature of MSC located in mouse incisors.

4.3.3 DPSCs and pulp regeneration

DPSCs are thought to reside in one or more specific niches, being activated and utilized in repair mechanisms following dental damage (Mitsiadis et al., 2011). In an adult mouse, incisors continuously wear away due to functional attrition. This attrition is counterbalanced by continuous generation of ameloblasts and odontoblasts from the stem cells that reside in the cervical loop area of the incisor apex (Harada, et al. 2002).

In adult Thy-1Cre^{+/+};R26R^{+/+} mice, LacZ⁺ cells traced during incisor development are lost due to incisor continuous growth. Accordingly, LacZ⁺ odontoblasts and those linked to blood vessels traced in adult incisor dental pulp are believed to come from the mesenchymal stem cell niche located at the apical end of the incisor. This is in agreement with other studies, in which the sublingual allogenic molar transplantation in LacZ transgenic R26R mice showed that the newly differentiated odontoblasts were exclusively derived from donor cells, which suggests that dental pulp has an undifferentiated lineage of cells that are able to regenerate pulp tissue (Takamori, et al. 2008).

Thy-1⁺ cells located in the blood vessel walls are especially intriguing. Activation of progenitor/stem cells in human immature third molars was studied using 5-bromo-2'-deoxyuridine labelling (BrdU). One day after deep cavity preparation BrdU labelling was strong in blood vessels surrounding the cavity, while after 4 days, the

labelling was restricted only to the cavity area. This demonstrates that perivascular progenitor/stem cells can proliferate in response to odontoblast injury and migrate to the injury site in their tissue of origin (Tecles et al., 2005). In addition, during incisor development and growth in NG2creER;R26R transgenic mice, pericytes were able to differentiate into odontoblasts (Feng et al., 2011). Thus, around 1% of LacZ⁺ odontoblasts are possibly derived from pericytes.

Collectively, LacZ⁺ cells in adult Thy-1Cre^{+/-};R26R^{+/-} mice were demonstrated in odontoblasts, subodontoblastic zone, perivascular area/blood vessels and some other dental pulp cells. This indicates that LacZ⁺ cells in adult Thy-1Cre^{+/-};R26R^{+/-} mouse incisors are derived from mesenchymal stem cells.

Chapter 5 : Regulation of Thy-1 Expression in Mouse Incisor Dental Pulp

5.1 Introduction

Mouse incisors are continuously erupting throughout life due to the presence of epithelial and mesenchymal stem cells at the apical end which proliferate and differentiate into ameloblasts and odontoblasts respectively (Harada et al., 1999). Sonic hedgehog (Shh) is a member of the mammalian Hedgehog (Hh) family that plays a key role during embryogenesis, organogenesis, and adult tissue homeostasis (King et al., 2008). Shh also influences stem cell behaviour in several tissues (Jiang and Hui, 2008) including teeth (Seidel et al., 2010b). This fact prompted us to investigate the effect of Shh on Thy-1 expression in mouse incisor dental pulp *in vitro* represented by cell and organ culture and *in vivo* using transgenic mice.

Shh signalling acts through the 12-pass transmembrane receptor Patched-1(Ptc-1) and the pseudo-G-protein coupled receptor Smoothened (Smo) membrane proteins, inducing the transcriptional activation of 3 target genes in vertebrates: the Glioblastoma (Gli) genes (Gli1, Gli2, and Gli3). In the absence of Shh, Ptc-1 maintains Smo in an inactivated state which is reversed after the binding of Shh, and the signal is transmitted to promote the transcription of Shh target genes, such as Ptc-1 and Gli-1 (Jiang and Hui, 2008, Kiefer, 2010, Mas and Ruiz i Altaba, 2010). So, in this study, Ptc-1 and Gli-1 were used as a read out of Shh signalling activity (Figure 5-1).

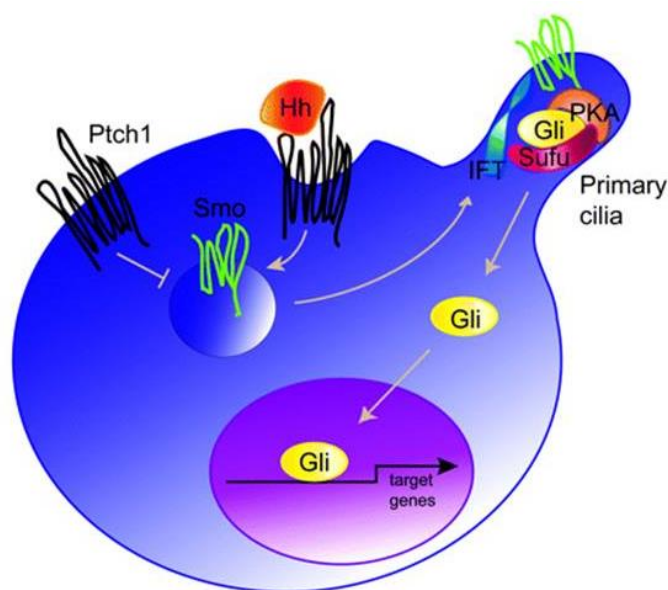


Figure 5-1: Shh signalling pathway. In the absence of Sonic Hedgehog (Shh) ligand, the pathway is inactive. Ptch1 inhibits the activity of Smo, thus the pathway activating Gli transcription factors is prevented from entering the nucleus and the Shh target genes are repressed. Activation of the pathway is initiated upon Shh binding to Ptch1, which leads to de-repression of Smo. As a consequence, a signalling cascade involving a multi-protein complex and the primary cilium as the processing platform leads to the translocation of the active form of Gli transcriptional activators to the nucleus. The Shh/Gli target genes are activated including Ptch1 and Gli1 itself. IFT, intraflagellar proteins; PKA, protein kinase A; Sufu, suppressor of fused (Adapted from (Kasper et al., 2009)).

Ptc-1^{fl/fl} transgenic mice were used to study the effect of Ptc-1 deletion on the developmental defects and embryonic lethal phenotype (Ellis et al., 2003). Also, Ptc-1 fl/fl in combination with Nestin Cre mice were used to study the role of Shh and Notch in regulating neurogenic divisions of neocortical progenitors (Dave et al., 2011).

Deletion of Ptc-1 leads to high levels of Shh target genes (Goodrich, et al. 1997), but homozygous inactivation of the Ptc-1 gene is embryonically lethal by E9.5 in mice (reviewed in Pazzaglia 2006). So, in this study, conditional inactivation of Ptc-1 has been accomplished in mice by the use of a Tamoxifen-inducible Cre-recombinase system, Ptc-1^{fl/fl};Cre ERTm (Zibat, et al. 2009).

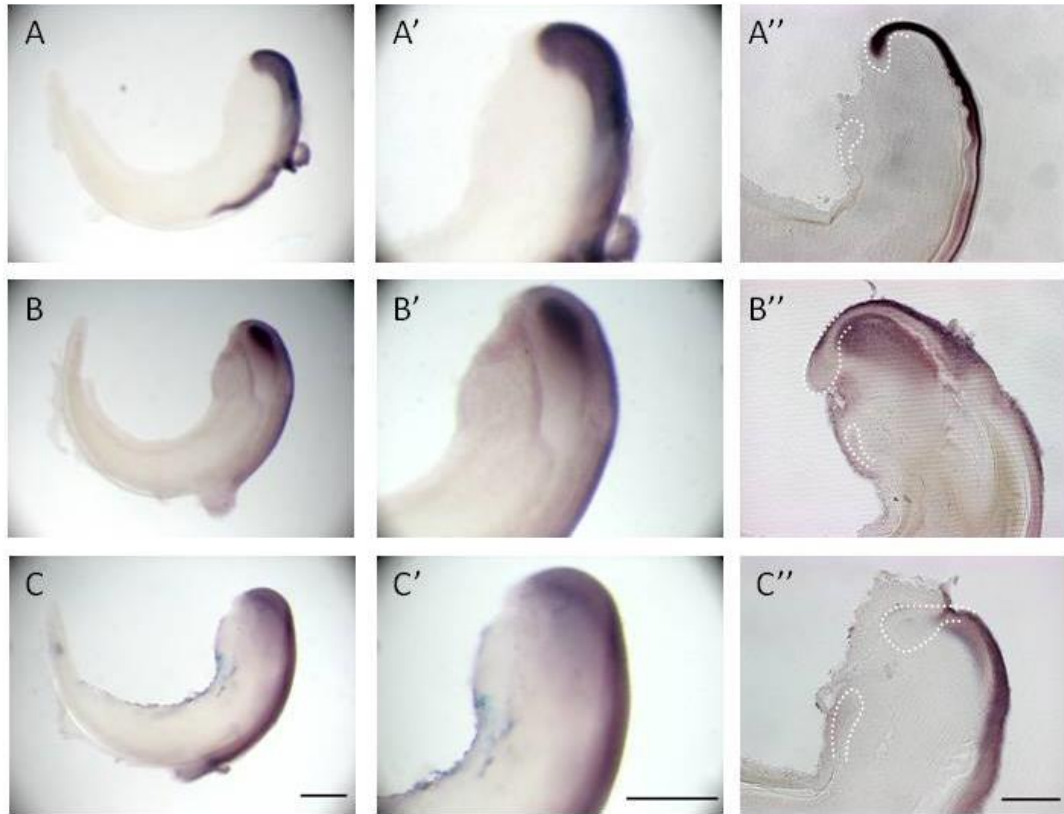
Primary cilia have recently been shown to play a crucial role in *Shh* signalling (Huangfu and Anderson 2005; Singla and Reiter 2006). Cilia have been reported to be present on dental epithelial and mesenchymal cells (Ohazama, et al. 2009) and human bone marrow MSCs (Tummala, et al. 2010). Polaris, the protein encoded by the *Tg737* gene, is a core component of the mammalian IntraFlagellar Transport (IFT) machinery and is required for the formation of all cilia and flagella (Pazour, et al. 2000). Loss of Polaris results in impaired *Shh* signalling pathway (Haycraft, et al. 2005). As in the case of *Ptc-1* null mutants, complete loss of *Polaris* function in *Tg737* mutants results in midgestation fatality (Murcia, et al. 2000). Thus, inducible deletion of Polaris is used to study its effect on developmental processes. To explore the role of Cilia in tooth development, Polaris was deleted from the tooth mesenchymal tissues using *Polaris*^{fl/fl} with Wnt-1 Cre transgenic mice (Ohazama et al., 2009). Also, Cartilage-specific mutant mice were created by mating *Col2a1*-Cre with Polaris-floxed mice to study the role of Cilia in cranial base development and growth (Ochiai et al, 2009). In this study *Polaris*^{fl/fl};Cre ERTm mice (Haycraft, et al. 2007) were used to study the effect of Polaris deletion on *Thy-1* expression in adult mouse incisor.

5.2 Results

5.2.1 *Shh*, *Ptc-1* and *Gli-1* are expressed in mouse incisor dental pulp

To explore the potential role of *Shh* signalling in Wild Type (WT) mouse incisor dental pulp tissue, we examined *Shh* expression along with the transcriptional effector genes (*Gli-1*) and Hh receptor gene *Patched* (*Ptc-1*) that are considered reliable indicators of Hh signalling activity (Ingham and McMahon, 2001). Their expression was investigated in PN5 mouse incisor dental pulp tissue using *in situ* hybridization (Figure 5-2).

Shh was localized to the labial dental epithelium. It was expressed in mature ameloblasts, premature ameloblasts, transient amplifying cells and the undifferentiated cells at the end of the labial cervical loop (Figure 5-2, A, A' and A''). Its receptor *Ptc-1* was expressed in both epithelial and mesenchymal tissues. In epithelial tissue, its expression was quite similar to *Shh* as it was expressed in the outer dental epithelium, ameloblast precursor cells and ameloblasts. Interestingly, in mesenchymal tissue, it was restricted to dental pulp mesenchyme next to the labial cervical loop where *Thy-1* was expressed (Fig 3.1). Also it was expressed in the mesenchymal tissue located between the two cervical loops and the mesenchymal tissue on the lingual side of lingual cervical loop (Figure 5-2, B, B' and B''). In contrast, expression of the downstream transcription activator *Gli-1* was higher in the epithelial tissues. It was expressed in the ameloblast precursor cells and ameloblasts, while its expression in the mesenchymal tissue was restricted to a narrow area next to the labial cervical loop in contact with the labial epithelium where the transient amplifying cells are located (Figure 5-2, C, C' and C'').



5-2: Expression of *Shh*, *Ptc-1* and *Gli-1* in PN5 mouse mandibular incisor by whole mount in situ hybridization (Sagittal sections). *Shh* is strongly expressed in the dental epithelium; the labial cervical loop, the ameloblasts precursor cells and the ameloblasts (A, A', A''). *Ptc-1* is expressed in outer dental epithelium, ameloblasts precursor cells, part of the inner dental epithelium and the mesenchymal tissue next to the bend of the labial cervical loop. Also it is weakly expressed in part of the mesenchymal tissue outside the lingual cervical loop and in between the two cervical loops (B, B', B''). *Gli-1* is expressed in ameloblasts precursor cells and the ameloblasts in addition to a narrow part of the mesenchymal tissue next to the labial cervical loop (C, C', C''). Labial and lingual cervical loops are highlighted with white dotted lines. Scale bar =500µm. These figures are representative for 3 independent experiments.

OPT was used to generate 3D mRNA expression of Shh, Ptc-1 and Gli-1 after detecting it using in situ hybridization. (Movies E, F and G respectively, included in the enclosed DVD). Representative images of the samples from the movie are shown in (Figure 5-3).

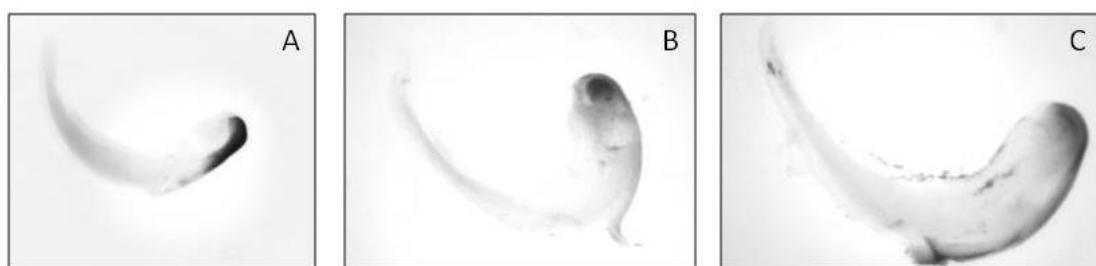


Figure 5-3: Still images of PN5 mouse mandibular incisor 3D construction after *in situ* hybridization. (A) Shh, (B) Ptc-1 and (C) Gli-1 were expressed in the apical area of the mouse incisor where the mesenchymal stem cells are located

The presence of Shh, Ptc-1 and Gli-1 mRNAs suggests that Shh signalling may play a developmental role in the postnatal mouse incisor.

5.2.2 Regulation of *Thy-1* expression *in vitro*

5.2.2.1 Shh does not affect *Thy-1* expression in cell culture

To evaluate the potential role of Shh signalling in *Thy-1* regulation in mouse dental pulp cell culture, *Ptc-1* and *Thy-1* double immuno-staining was performed for PN5 mouse digested dental pulp tissues. Flow cytometry analysis showed that almost 20% of dental pulp cells were positive for both *Ptc-1* and *Thy-1* which means that some of *Thy-1*⁺ cells are receptive for Shh signalling (Figure 5-4, A).

Cells were stimulated with 100nM Smoothened Agonist (SAG) (a chlorobenzothiophene-containing Hh pathway agonist) (Chen et al., 2002b) and 10 μ M Cyclopamine (a steroidal alkaloid previously shown to block Shh signalling (Hh antagonist)) (Martínez et al., 2011). Both of them act by binding directly to the Smoothened receptor (Smo) (Chen et al., 2002a). FACS results analysis showed that after 24 hours cell incubation with SAG, and 48 hours incubation with Cyclopamine, *Ptc-1*, the read-out gene, showed an increased expression in the presence of SAG from 15% to 23% (Figure 5-4, B and D) and a decrease in the presence of Cyclopamine from 15% to 7% (Figure 5-4, C and E). Surprisingly *Thy-1* expression remained unaffected in both cases (Figure 5-4, B, C, D, and E).

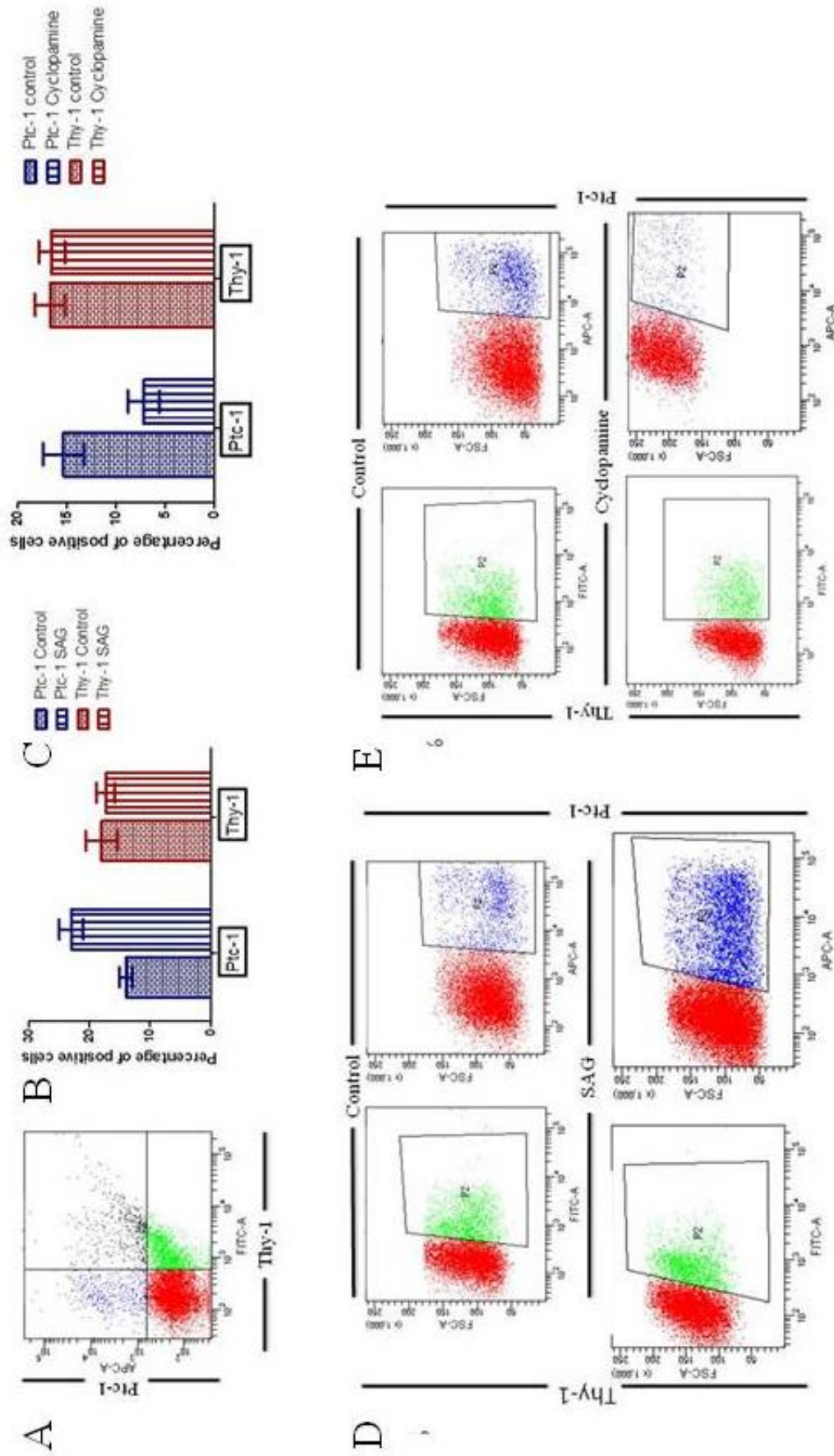


Figure 5-4: Effect of Shh agonist and antagonist on Thy-1 PN5 mouse incisor dental pulp cell culture. (A) Thy-1 and Ptc-1 double staining showing about 5% of dental pulp cells express Thy-1 and Ptc-1. (B, D) Ptc-1 expression increased with SAG while Thy-1 expression did not. (C, E) Ptc-1 decreased with Cyclopamine while Thy-1 expression remained the same. Error bar=mean±SD. P2 represents dental pulp tissue cells that express positive levels of FITC (green) for Thy-1 and APC (blue) for Ptc-1. The controls included IgG2b FITC and IgG APC stained dental pulp cells. These results are representative for 3 independent experiments.

5.2.3 Shh regulates Thy-1 expression in organ culture

To further explore the role of Shh in Thy-1 regulation during incisor development, an *in vitro* organ culture system was established in which Thy-1 expression could be assayed. PN5 incisor explants were cultured for 24 and 48 hours with the signalling activator and inhibitor respectively. Then, they were assayed using whole mount *in situ* hybridization.

To assess the incisor explant culture system, PN5 incisors were cultured for 48 hours in α MEM media and Thy-1 expression was then assayed. After hybridization with a Thy-1 probe, Thy-1 expression was noted in the apical mesenchymal tissue, similar to normal expression of Thy-1 observed in uncultured mandibular incisors (Figure 5-5). This indicates that this incisor explant system was suitable for the study of Thy-1 regulation.

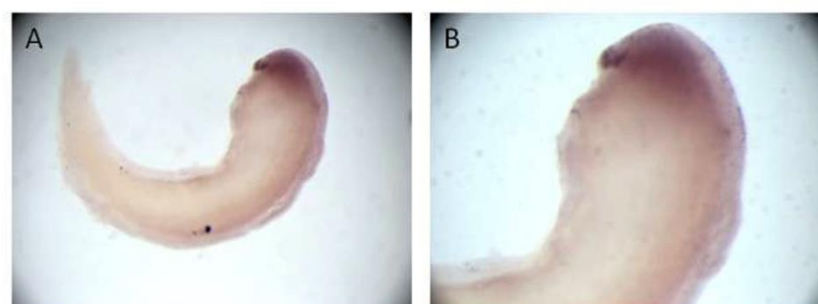


Figure 5-5: Expression of Thy-1 in a cultured mandibular incisor of PN5 wild type mouse. Thy-1 expression still restricted to the mesenchymal tissue adjacent to the labial cervical loop after being cultured for 48 hours.

5.2.3.1 *Thy-1* expression is up-regulated in the presence of Shh signalling pathway activator in mouse incisors

To investigate the effect of increasing Shh signalling on *Thy-1* expression in mouse incisors, the chemical activator (SAG) was applied to incisor organ culture. Its concentration was chosen based on previous studies in our laboratory. Since the chemical activator was dissolved in water, an equal volume of water served as a control treatment in the activator experiment.

Activation of Shh signalling by incubating incisors with 200nM SAG for 24 hours resulted in up-regulation of *Ptc-1* expression in the incisor dental pulp tissue (Figure 5-6, A, A', n=3) compared to *Ptc-1* expression in the water control explants (Figure 5-6, B, B', n=3).

Similarly, adding SAG to the culture resulted in an increase of *Thy-1* expression (Figure 5-7, A, A', n=3) when compared to their water treated control (Figure 5-7, B, B', n=3). This indicates that activation of Shh signalling pathway affects *Thy-1* expression in mouse incisor organ culture.

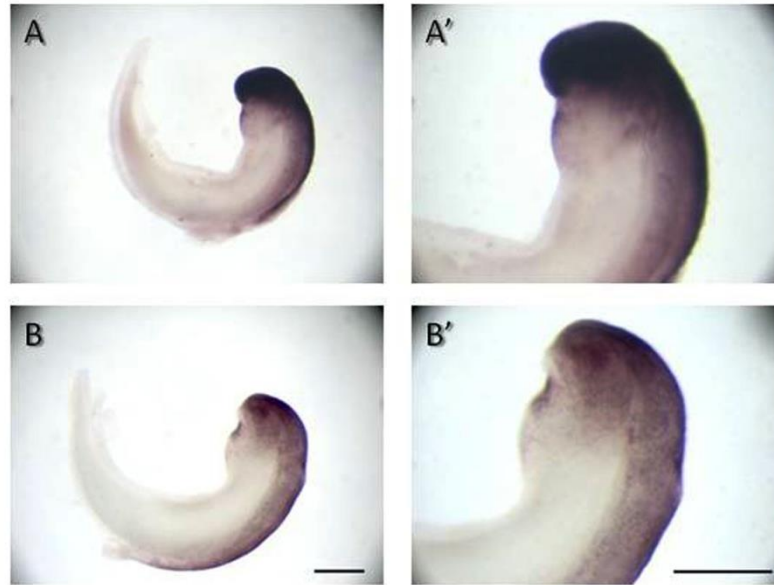


Figure 5-6: Control experiment for SAG, an activator of Shh signalling. (A, A') Up-regulation of Ptc-1 in PN5 mandibular incisor explants cultured for 24 hours with a culture medium containing 200 nM SAG (n=3) compared to water treated controls (B, B') (n=3). Scale bar=500µm. These figures are representative of 3 independent experiments.

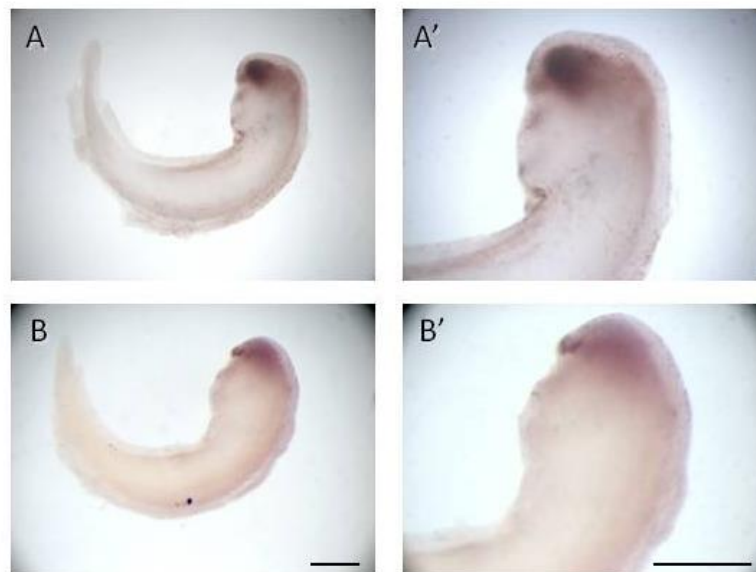


Figure 5-7: Effect of SAG (Shh signalling activator) on Thy-1 expression in PN5 mouse mandibular incisor. Thy-1 expression was up-regulated in the mouse incisor dental pulp incubated with SAG (n=3) (A, A') when compared to the control ones (n=3) (B, B'). Scale bar =500µm.

5.2.3.2 *Thy-1* expression is down-regulated in the presence of *Shh* signalling pathway inhibitor in mouse incisors.

To investigate the effect of decreasing *Shh* signalling on *Thy-1* expression in mouse incisors, *Shh* signalling inhibitor Cyclopamine (Cyc) was added to the incisor organ culture media. The chemical inhibitor was dissolved in 100% ethanol, an equal volume of ethanol served as a control treatment in the inhibition experiment. Incubation of PN5 mandibular incisors with 20 μ M (Cyc) (Nagase *et al.*, 2005) for 48 hours resulted in a marked down-regulation of *Ptc1*, a downstream target gene of *Shh* (Figure 5-8, A, A' $n=3$), compared to strong *Ptc1* expression in the ethanol control explants (Figure 5-8, B, B', $n=3$).

Incubation of PN5 mandibular incisor explants cultured with similar dose of Cyc resulted in a decrease of *Thy-1* expression (Figure 5-9, A, A') when compared to their ethanol treated controls (Figure 5-9, B, B'). This demonstrated the effect of adding Cyc on *Thy-1* expression in mouse incisor organ culture.

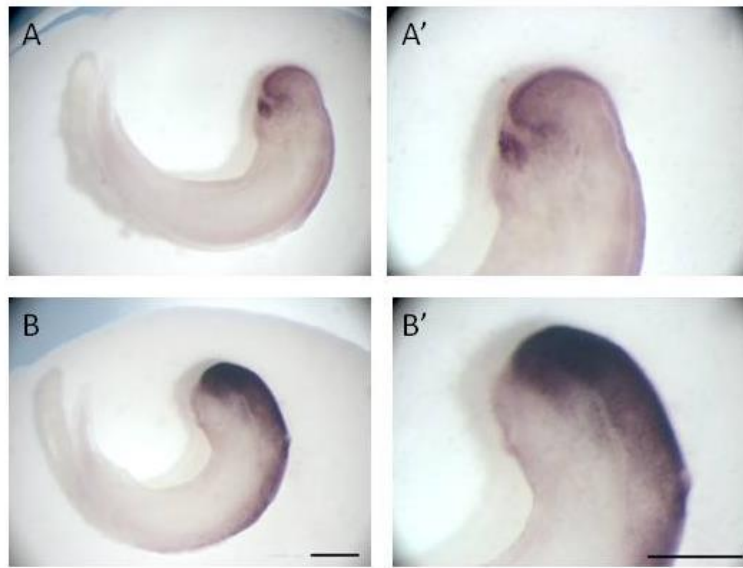


Figure 5-8: Control experiment for Cyclopamine, an inhibitor of Shh signalling. (A, A') Down-regulation of *Ptc-1* in PN5 mandibular incisor explants cultured for 48 hours with a culture medium containing 20 μ M Cyclopamine (n=3) compared to ethanol treated controls (n=3) (B,B'). Scale bar=500 μ m. These figures are representative of 3 independent experiments.

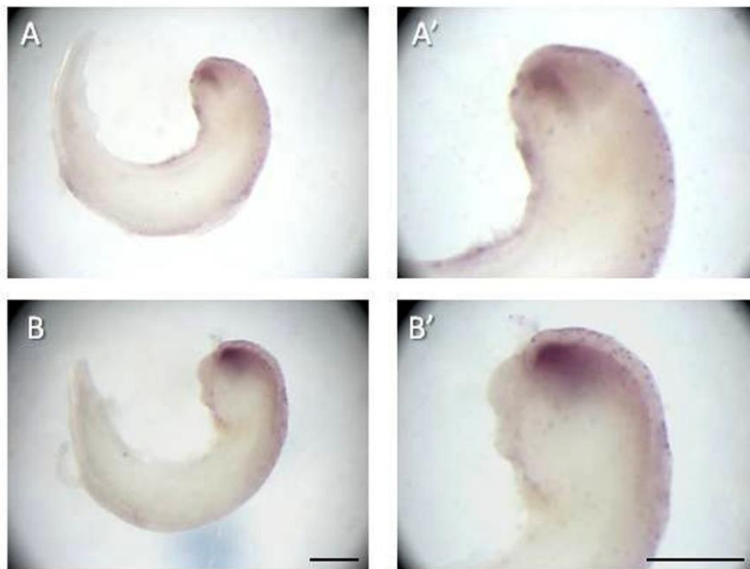


Figure 5-9: Effect of Cyclopamine, Shh signalling inhibitor on *Thy-1* expression in PN5 mouse mandibular incisors. (A) Down-regulation of *Thy-1* expression in PN5 mandibular incisor explants cultured for 48 hours in the presence of 20 μ M cyclopamine (n=3) compared to ethanol treated controls (n=3) (B). Scale bar=500 μ m. These figures are representative of 3 independent experiments.

5.2.4 *Thy-1* expression in *Ptc-1^{fl/fl};CreERTm* transgenic mice

Tamoxifen was administered as mentioned in details in Materials and Methods .

5.2.4.1 *Thy-1* expression is up regulated in *Ptc-1^{fl/fl};CreERTm* transgenic mice

To further investigate the regulation of *Thy-1* *in vivo*, its expression was investigated in *Ptc-1^{fl/fl};CreERTm* adult mouse incisor by *in situ* hybridization. *Gli-1* expression was examined as a read-out of *Shh* signalling pathway activity. Similar to PN5 mice, *Gli-1* was expressed in the epithelial and mesenchymal tissues. In corn-oil treated mutant mice (control), *Gli-1* was expressed in the labial cervical loop, ameloblasts precursors, and ameloblasts. It was also expressed in the mesenchymal tissue adjacent to the labial cervical loop and slightly in the ameloblasts layer with weak expression at and adjacent to the lingual cervical loop (Figure 5-10, A, A' and A'',n=3).

In Tamoxifen (Tam) treated mutant mice, *Gli-1* expression was increased when compared to the corn-oil treated controls indicating *Shh* signalling pathway activation. *Gli-1* was expressed in the same areas as control mice but with more intensity (Figure 5-10, B, B' and B'',n=3).

Interestingly, *Thy-1* expression was up regulated in *Ptc-1^{fl/fl};CreERTm* Tam treated adult mouse incisor dental pulp tissues when compared to its expression in the same tissues of the corn-oil treated controls (Figure 5-11, B, B' and B'',n=3). In control mice, *Thy-1* was expressed in the mesenchymal tissue next to the labial cervical loop, subodontoblastic zone with a very weak expression related to the lingual cervical loop. (Figure 5-11, A, A' and A'',n=3).

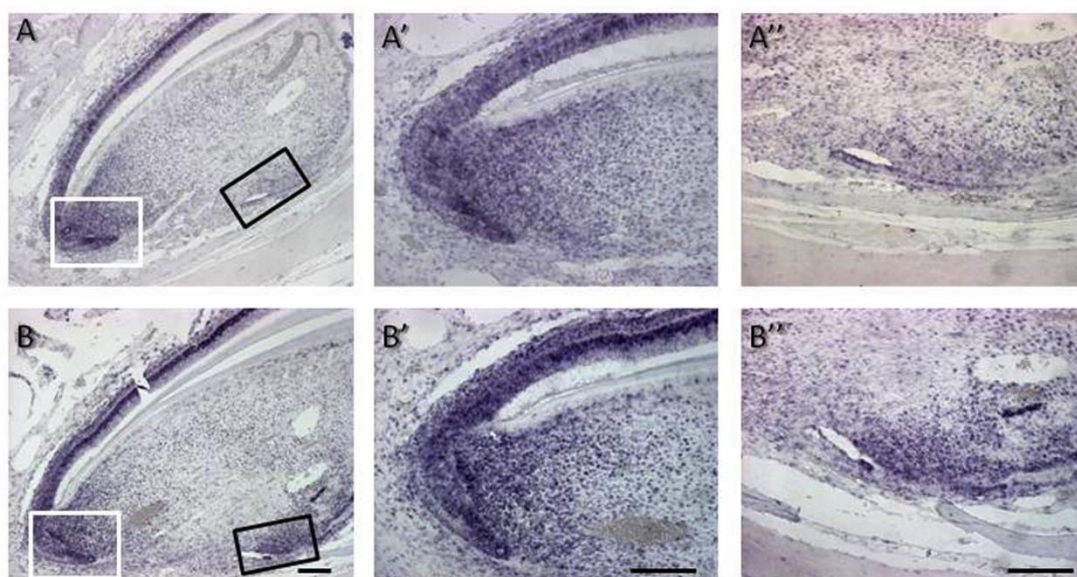


Figure 5-10: Gli-1 expression in adult mouse mandibular incisor dental pulp of corn-oil and Tamoxifen treated *Ptc-1^{fl/fl};CreERTm* mice (Sagittal sections). (A) Gli-1 was expressed in both; epithelial and mesenchymal tissues in the adult mouse dental pulp (A). (A') Higher magnification of the white-boxed area shows Gli-1 expression in the mesenchymal tissue next to the labial cervical loop and the ameloblasts precursors and ameloblasts. (A'') Higher magnification of the black-boxed area shows Gli-1 weak expression at the lingual cervical loop and the mesenchymal tissue next to it. (B) Gli-1 expression was up-regulated in the Tamoxifen treated mice. (B') Higher magnification of the white-boxed area shows intensified expression of Gli-1 in labial cervical loop, ameloblasts precursors, ameloblasts and mesenchymal tissues next to it. (B'') Higher magnification of the black-boxed area shows higher expression of Gli-1 in the lingual cervical loop and the mesenchymal tissue next to it. Scale bar = 500µm. These figures are representative of 3 independent experiments.

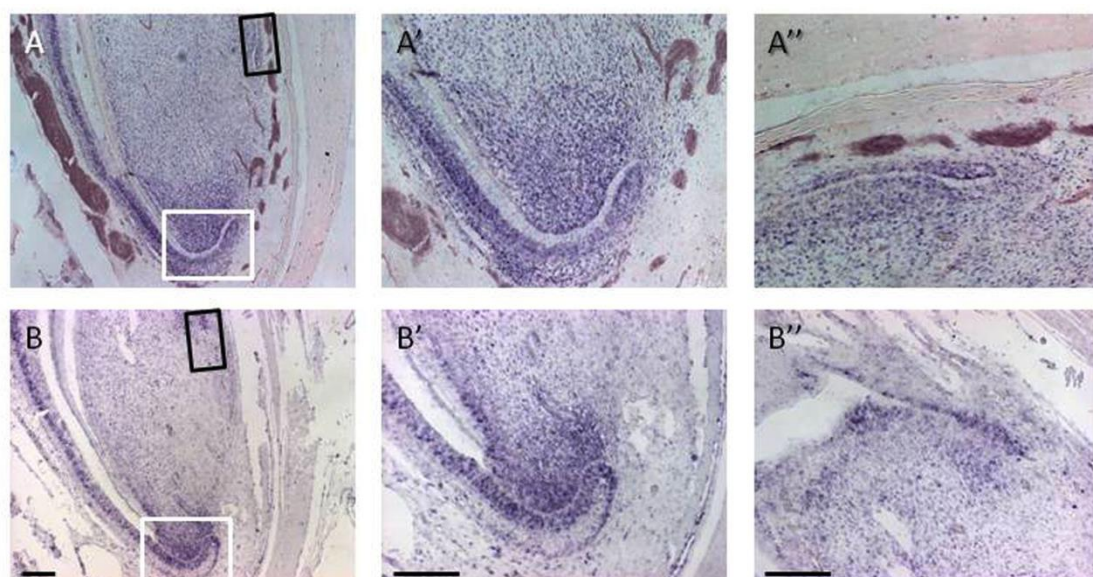


Figure 5-11: Thy-1 expression in adult mouse mandibular incisor dental pulp tissues of Tamoxifen and corn-oil treated *Ptc-1^{fl/fl};CreERTm* mice (Sagittal sections). (A) Shows Thy-1 expression in the dental pulp of corn-oil treated mice (A). (A') Higher magnification of the white box in (A) shows Thy-1 expression in the mesenchymal tissue next to the labial cervical loop. (A'')

Higher magnification of the black box shows very weak Thy-1 expression in the mesenchyme next to lingual cervical loop. (B) shows Thy-1 expression up regulation in Tamoxifen treated mice. (B') Higher magnification of the white box in (B) shows increased Thy-1 expression at the mesenchymal tissue adjacent to the labial cervical loop (B'') Higher magnification of the black box in (B) shows higher expression of Thy-1 the mesenchymal tissue next to lingual cervical loop. Scale bar =500µm. These figures are representative of 3 independent experiments.

Histologically, the maxillary and mandibular incisors of Tam treated Ptc-1^{fl/fl};Cre ERTm and corn-oil treated control mice were normal. They both showed normal curvature and length of labial and lingual cervical loops. Ameloblasts and odontoblasts were normal in development, differentiation, shape and number (Figure 5-12).

In order to evaluate the incisor length, measurement analysis was carried out as described in section 2.12 The mean lengths of mandibular incisors of Ptc-1^{fl/fl};CreER^{Tm-} and Ptc-1^{fl/fl};CreER^{Tm+} mice were 11.7±1 mm and 9.7±2 mm respectively, and the mean length of maxillary incisors of corn oil and Tam treated mice were 7.4 ±0.5 and 7.1±0.7 mm respectively. Statistical analysis of (student's *t*-test) additionally revealed that neither maxillary nor mandibular incisors of Ptc-1^{fl/fl}; CreER^{Tm+} were significantly shorter than those of Ptc-1^{fl/fl}; CreER^{Tm-} ones (Figure 5-13).

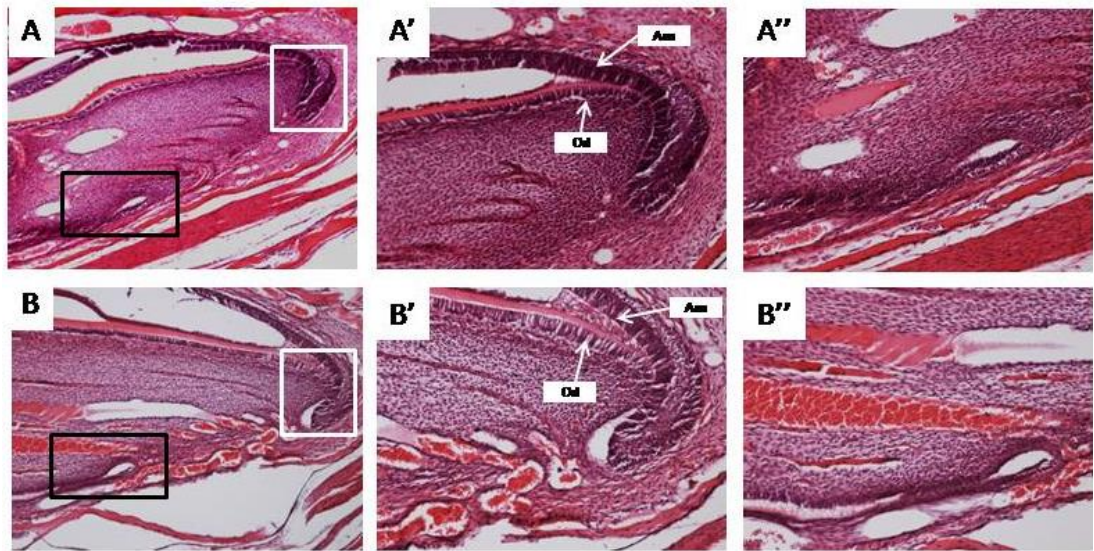


Figure 5-12: Hematoxylin and Eosin stained sagittal sections of mandibular incisors of adult *Ptc-1*^{fl/fl}; *CreERT^m* mice. (A) and (B) show normal cervical loop area in *Ptc-1*^{fl/fl}; *CreERT^m* corn-oil and Tamoxifen treated mice respectively. White and Black boxed areas in (A) and (B) magnified in (A'), (A''), (B') and (B'') showing the labial and lingual cervical loops respectively. (A') and (B') show normal development of ameloblasts (Am) and odontoblasts (Od). Scale bar = 500µm.

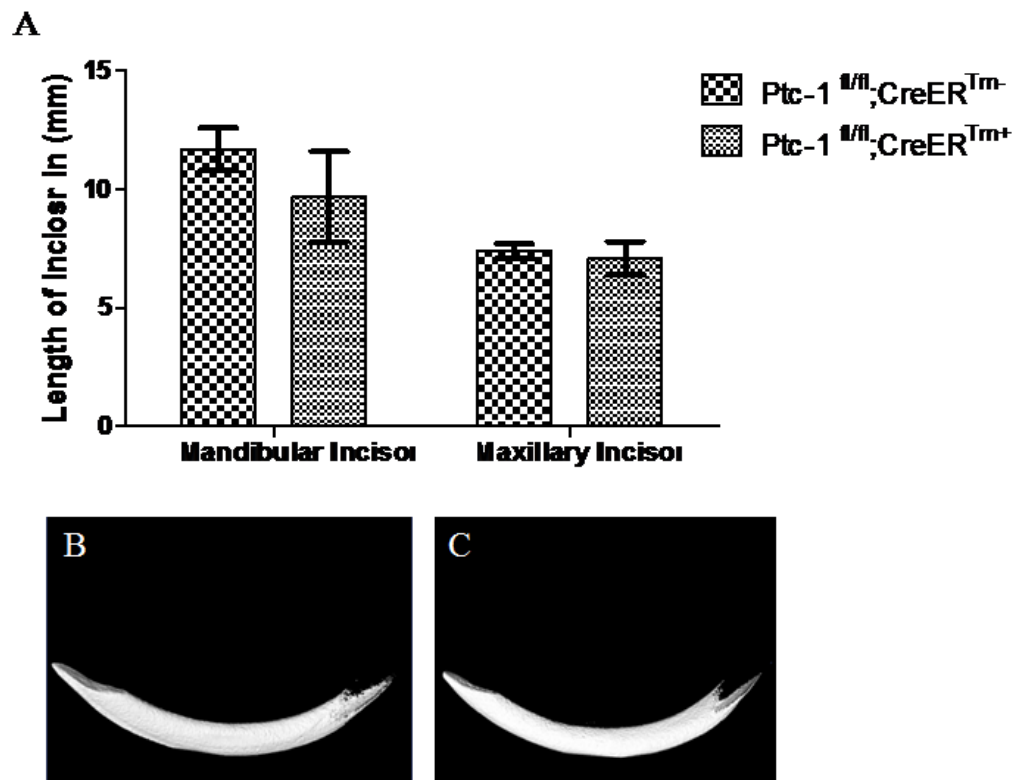


Figure 5-13: Micro CT analysis of *Ptc-1*^{fl/fl}; *CreERT^m*⁻ and *Ptc-1*^{fl/fl}; *CreERT^m*⁺ incisors. (A) Mean length ± SD of mandibular and maxillary of *Ptc-1*^{fl/fl}; *CreERT^m*⁻ (n=3) and *Ptc-1*^{fl/fl}; *CreERT^m*⁺ (n=3). There were no significant difference between the length of incisors according to Student's *t*-test (*p* < 0.05).

0.05). (B) $Ptc-1^{fl/fl};CreER^{Tm+}$ incisor appears to be normal similar to $Ptc-1^{fl/fl};CreER^{Tm-}$ incisor (C). This figure is representative for 3 independent measurements.

In conclusion, in Tam treated $Ptc-1^{fl/fl};CreER^{Tm}$ mice, *Gli-1* and *Thy-1* expression were up-regulated in mouse incisor dental pulp. Tam treated mouse incisors were slightly shorter when compared to the corn-oil treated controls. However, this difference was not significant. Also, there was no histological difference between the two groups.

5.2.5 *Thy-1* expression in *Polaris*^{fl/fl};CreERTm transgenic mice

5.2.5.1 *Thy-1* expression was down regulated in *Polaris*^{fl/fl};CreERTm transgenic mice

Primary cilia are solitary non motile microtubule based organelles emerging from the distal centriole of the centrosome of many mammalian cells (Wheatley et al., 1996). Hedgehog signalling which controls crucial aspects of development and stem cells function is regulated through the transport of key signalling proteins into and out of the primary cilium (Rohatgi et al., 2007). *Polaris* is one of the proteins that are essential for cilia formation and hedgehog signalling. Its deletion results in absence of cilia (Zhang et al., 2003) and causes defects in hedgehog signalling (Liu et al., 2005). In a similar experiment to *Ptc-1*^{fl/fl};CreERTm transgenic mice, *Thy-1* expression was investigated in *Polaris*^{fl/fl};CreERTm transgenic mice. Again, *Gli-1* expression was examined as an indicator of Shh signalling pathway activity.

Gli-1 expression was decreased in Tam treated *Polaris*^{fl/fl};CreERTm mice compared to the corn-oil treated controls. In *Polaris*^{fl/fl};CreERTm corn-oil treated mice, *Gli-1* was expressed in the same pattern similar to *Ptc-1*^{fl/fl};CreERTm mice. It was expressed in the mesenchymal tissue next to the labial cervical loop in the ameloblast precursors and ameloblasts. Similarly it had a weak expression in and next to the lingual cervical loop (Figure 5-14, A, A' and A'', n=3).

In Tam treated *Polaris*^{fl/fl};CreERTm mice, *Gli-1* expression was strongly down-regulated. It had a very weak expression in the mesenchymal tissue adjacent to the labial cervical loop. Also it showed faint expression in ameloblasts precursors and ameloblasts with no expression at the lingual cervical loop or in the adjacent mesenchymal tissue (Figure 5-14, B, B' and B'', n=3).

In *Polaris^{fl/fl};CreERTm* control mice, *Thy-1* was expressed in the mesenchymal tissue next to the labial cervical loop, in the subodontoblastic layer, lingual cervical loop and the mesenchymal tissue adjacent to it (Figure 5-15, A, A' and A'', n=3). Correspondingly like *Gli-1* expression, *Thy-1* expression was slightly down-regulated in *Polaris^{fl/fl};CreERTm* Tam treated mice. It showed weak expression in both the mesenchymal or epithelial tissues (Figure 5-15, B, B' and B'', n=3).

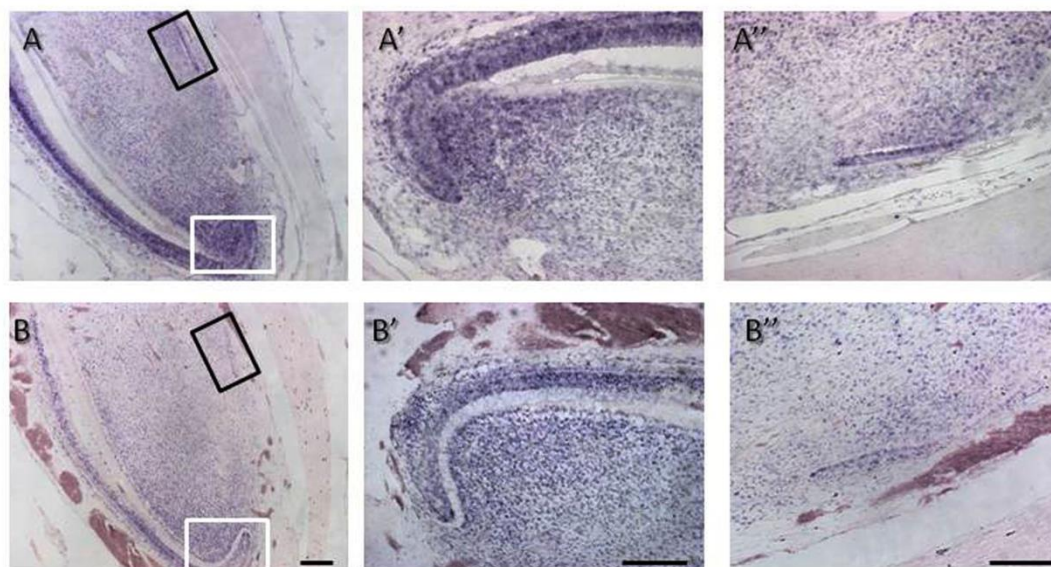


Figure 5-14: Gli-1 expression in the adult mouse mandibular incisor dental pulp of adult Tamoxifen and corn-oil treated *Polaris*^{fl/fl};CreERTm mice (sagittal section). In corn-oil treated mice, Gli-1 was expressed in the dental epithelial and mesenchymal tissues (A). (A') Higher magnification of the white boxed area in (A) shows Gli-1 expression in the labial cervical loop, ameloblasts precursors, ameloblasts and the mesenchymal tissues next to it. (A'') Higher magnification of the black-boxed area in (A) shows Gli-1 weak expression in the lingual cervical loop and the mesenchymal tissue adjacent to it. In Tamoxifen treated mice, Gli-1 expression was strongly down-regulated (B). (B') Higher magnification of the white boxed area in (B) shows very weak Gli-1 expression in the mesenchymal tissue next to the labial cervical loop, ameloblasts precursors and ameloblasts. (B'') Higher magnification of the black-boxed area in (B) shows no expression for Gli-1 in the lingual cervical loop or the mesenchymal tissues next to it. Scale bar = 500µm. These figures are representative of 3 independent experiments.

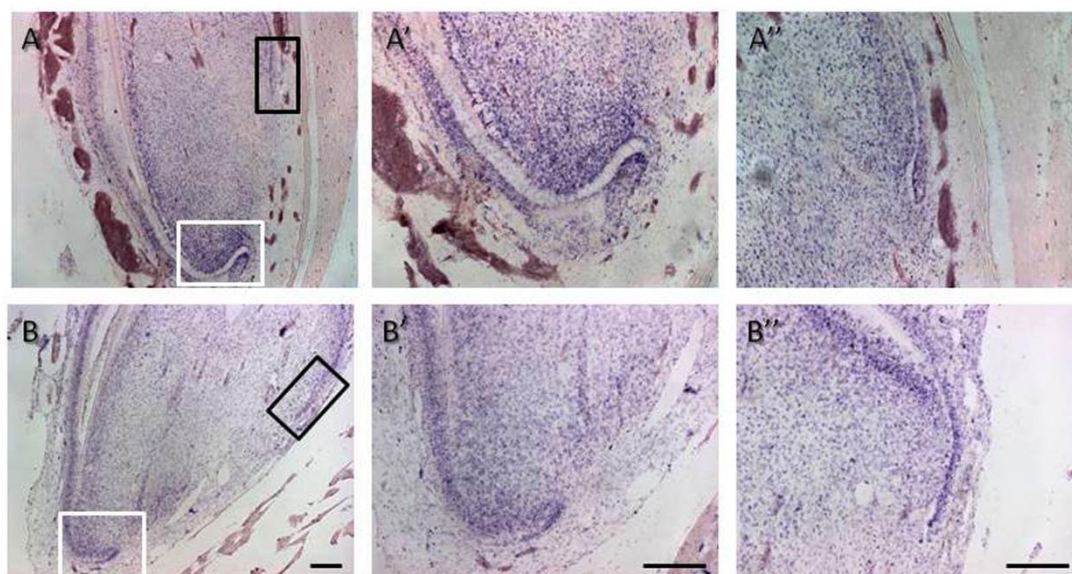


Figure 5-15: Thy-1 expression in the mandibular incisor dental pulp of adult corn-oil and Tamoxifen treated *Polaris*^{fl/fl};CreERTm mice (Sagittal section). In corn-oil treated mice, Thy-1 was

expressed in the apical mesenchymal tissue next to the labial cervical loop (A). (A') Higher magnification of the white boxed area in (A) shows *Thy-1* expression in the mesenchymal tissue adjacent to the labial cervical loop. (A'') Higher magnification of the black boxed area in (A) showing *Thy-1* expression close to lingual cervical loop. In Tamoxifen treated mice, (B) *Thy-1* expression was decreased slightly in mesenchymal tissue adjacent to both labial cervical loop (B') and lingual cervical loop (B''). Scale bar= 500µm. These figures are representative of 3 independent experiments.

Histologically, the mandibular incisors of Tamoxifen treated *Polaris^{fl/fl};CreERTm* mice were as normal as their corn-oil treated controls. They showed normal curvature and length of both, labial and lingual cervical loops. Ameloblasts and odontoblasts were normal in development, differentiation, shape and number (Figure 5-16).

To evaluate the incisor length, measurement analysis of incisor length was carried out as described in section 2.12. The mean lengths of mandibular incisors of *Polaris^{fl/fl};CreER^{Tm-}* and *Polaris^{fl/fl};CreER^{Tm+}* mice were similar, both of them equal to 14±0.5 mm, whereas the mean length of maxillary incisors of corn-oil and Tam treated mice were 8.5±0.7 and 8.4±0.7 mm respectively. Statistical analysis (student's t-test) additionally revealed that both maxillary and mandibular incisors of *Polaris^{fl/fl};CreER^{Tm+}* were not significantly different from those of *Polaris^{fl/fl};CreER^{Tm-}* incisors (Figure 5-17).

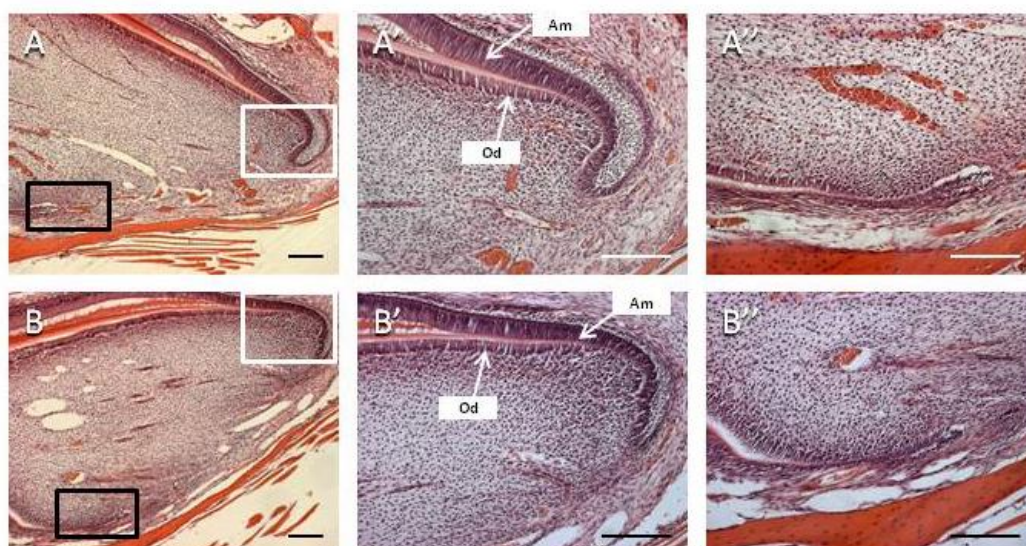


Figure 5-16: Heamatoxylin and Eosin stained sagittal sections of mandibular incisors of adult *Polarix*^{fl/fl}; *CreER*Tm mice. (A) and (B) show a normal cervical loop area in *Polarix*^{fl/fl}; *CreER*Tm corn-oil and Tamoxifen treated mice respectively. White and Black boxed areas in (A) and (B) magnified in (A'), (A''), (B') and (B'') show the labial and lingual cervical loops respectively. (A') and (B') show normal development of ameloblasts (Am) and odontoblasts (Od). Scale bar = 500μm.

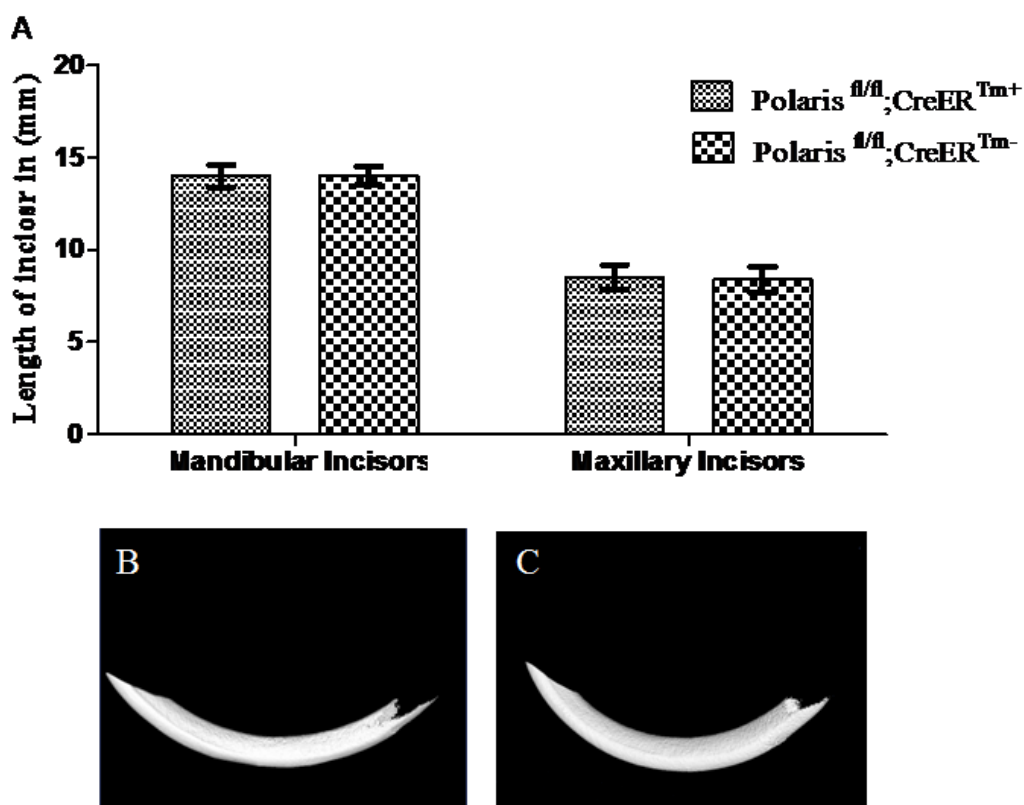


Figure 5-17: Micro CT analysis of *Polarix*^{fl/fl}; *CreER*^{Tm-} and *Polarix*^{fl/fl}; *CreER*^{Tm+} incisors. (A) Mean length \pm SD of mandibular (mand) and maxillary (max) of *Polarix*^{fl/fl}; *CreER*^{Tm-} ($n=3$) and *Polarix*^{fl/fl}; *CreER*^{Tm+} ($n=3$). There were no significant differences between the length of incisors according to Student's *t*-test ($p < 0.05$). (B) *Polarix*^{fl/fl}; *CreER*^{Tm+} incisor appears to be normal and similar to *Polarix*^{fl/fl}; *CreER*^{Tm-} incisor (C). This figure is representative of 3 independent measurements.

Collectively, in Tam treated Polaris^{fl/fl};CreERTm mice, Gli-1 and Thy-1 expression were down-regulated compared to the corn-oil treated mice. Nevertheless, both groups showed normal histology and equal incisor length.

5.3 Discussion

5.3.1 *Shh* expression in mouse incisor

Shh, an important morphogen, is involved in a variety of cellular processes during development and diseases, including cell-fate determination, proliferation, and differentiation (Cobourne et al., 2009). Recent studies identified involvement of *Shh* in the proliferation and cell-fate specification of several stem cells such as neural stem cells and mesenchymal stem cells (Kondo et al., 2005, Palma et al., 2005). To test whether *Shh* could have a role in *Thy-1* expression regulation in mouse incisors, we first analyzed the expression of *Shh*, *Ptc-1* and *Gli-1* in WT mouse incisor dental pulp tissues. *Ptc-1* and *Gli-1* are *Shh* targets and reliable markers of *Shh* signalling pathway activity (Lee et al., 1997). At E15.5, *Shh* was known to be expressed in epithelial cells on the labial side near the labial cervical loop (Klein et al., 2008), and its protein was detected in the pre-ameloblasts and the mesenchymal cells adjacent to the labial cervical loop in adult mice (Seidel et al., 2010b). In PN5 mouse incisors, *Shh* mRNA was detected in epithelial cells in the labial cervical loop and pre-ameloblast cells, which indicates that *Shh* expression in the mouse incisor is maintained in the same tissues throughout different stages in tooth development. This suggests the important and different roles of *Shh* in the growing mouse incisor.

During tooth development, *Ptc-1* and *Gli-1* were expressed similarly in the odontogenic epithelium and mesenchyme of the incisors (Hardcastle et al., 1999). In PN5 mice in this study, *Ptc-1* and *Gli-1* maintained this similarity as both were expressed in the epithelial cells of the labial cervical loop and the mesenchymal tissue adjacent to it.

5.3.2 *Thy-1* expression is regulated by Shh in mouse incisor organ culture but not dental pulp cells in culture.

The question of whether exogenous Shh signalling regulates *Thy-1* expression was determined by adding an Hh activator (SAG) or inhibitor (Cyc) to the culture media in organ and cell culture. Incisor tooth germs of PN5 mice were used in organ culture as they could be dissected out from calcified mandibular bone to make them more permeable to the media in the culture.

A selection of control experiments for the chemical activation and inhibitor of Shh were used in the study to ensure their efficiency and working concentrations in tooth tissues. Application of Shh protein on beads showed that downstream genes in the pathway could be activated in mesenchyme (Hardcastle, et al. 1999). The results of the organ culture experiments showed that *Thy-1* was up-regulated and down-regulated by (SAG) and (Cyc) respectively. These results imply that *Thy-1* is regulated through the transcriptional factor Gli-1 which indicates that *Thy-1* might be a downstream target of this transcription factor.

Shh expression has been previously shown to be highly restricted to epithelial cells during tooth development (Bitgood and McMahon, 1995). In dental pulp cell culture, the dental epithelium was removed and consequently the endogenous source of Shh was eliminated. Shh producing feeder layers and Cyc were able to stimulate and inhibit the proliferation of human putative epidermal stem cells respectively (Zhou et al., 2006). However, in the same study, recombinant Shh-N fragmented at different concentrations and even at 500ng/ml was not able to increase cell proliferation. In this study, *Thy-1* expression was not affected by SAG or Cyc in cell culture even though *Ptc-1* was up-regulated and down-regulated respectively.

To sum up, the gene expression analysis and *in vitro* experiments indicate that Shh signalling does not affect the expression of *Thy-1* in culture. Nevertheless, it has affected *Thy-1* expression in organ culture. This could explain why *Thy-1* regulation, in addition to the Shh signalling pathway, may rely on the epithelial mesenchymal interaction which is intact in organ culture but is disrupted in the cell culture system.

5.3.3 *Thy-1* expression is affected by Shh signalling *in vivo* in adult mouse incisors

5.3.3.1 *Thy-1* expression is up-regulated in *Ptc-1*^{fl/fl};Cre ERTm transgenic mice

In the absence of *Ptc-1*, the Hh pathway is fully activated (Huangfu and Anderson, 2005). In *Ptc-1*^{fl/fl};CreERTm mice, *Gli-1* expression and *Thy-1* in Tam treated mice were up-regulated when compared to their expression in controls although they were expressed in the same tissues. *Gli-1* up-regulation indicates the Shh signalling pathway increased activity. *Thy-1* up regulation indicates that it is affected by Shh signalling pathway activation *in vivo* and it may lie downstream to *Gli-1* transcription factor.

Histologically, the mouse incisors showed no difference between the experimental and the control groups with ameloblasts and odontoblasts showing normal differentiation and polarization. X-ray CT scans showed that there was no significant difference between mouse incisors length in Tam treated and control mice

5.3.3.2 *Thy-1* expression is down regulated in *Polaris*^{fl/fl};CreERTm transgenic mice

The requirement for the cilium in Hedgehog signalling was first realized after the identification of mouse mutants that disrupt the intraflagellar transport proteins, IFT88 and IFT172 (Huangfu et al., 2003). Since then, a number of regulators of

intraflagellar transport genes have been shown to be required for Shh signalling such as Ift144, Ift 122, Ift172 and Ift52. (Wong and Reiter, 2008) . In addition, several components of the Shh pathway components are present within the cilium.

Polaris, the protein encoded by *Tg737* gene, is a core component of the mammalian IFT machinery and is required for the formation of all cilia and flagella (Murcia et al., 2000). Mice homozygous for the hypomorphic *Tg737* Oak Ridge Polycystic Kidney (*orpk*) allele exhibit phenotypes in many tissues including the formation of cysts in the kidney, liver, and pancreas, hydrocephalus, and skeletal patterning defects that include extra molar teeth, cleft palates, and pre axial polydactyly (Moyer et al., 1994, Yoder et al., 2002, Zhang et al., 2003). While *Tg737^{orpk}* mutants are viable, complete loss of *Tg737* function in *Tg737* mutants results in mid gestation lethality, randomization of the left–right body axis, neural tube closure, patterning defects and formation of eight to ten un-patterned digits per limb (Murcia et al., 2000). Genetic studies indicated that IFT are required for sonic hedgehog signalling downstream of *Smo* and *Ptc-1* but upstream of the *Gli* transcription factors (Haycraft et al., 2005). During tooth development, *Tg737* was expressed and cilia were present throughout the dental epithelium and in the underlying mesenchyme, as determined by expression of β -gal in embryos heterozygous for the *Tg737 ^{Δ 2-3 β -gal}* allele (Ohazama et al., 2009).

In, *Ift88^{fxo/-}* (Hypomorphic mutations that partially disrupt anterograde IFT, Polaris), E9.5 embryo neural tubes and limb buds showed partial reduction of *Shh* pathway activation level which resulted in a weaker *Gli-1* and *Ptch-1* compared to the wild type mice (Liu et al., 2005). In our study, *in situ* hybridization demonstrated that the

expression of *Gli-1* was down-regulated in Tam treated *polaris^{fl/f};CreERTm* mice confirming the reduction of *Shh* pathways in these mice. *Thy-1* expression was also down-regulated which confirms that *Thy-1* lies downstream to *Gli-1* and is to be set by *Gli-1* activator (*GliAct*) forms rather than *Gli-3* repressor (*Gli3Rep*) (te Welscher et al., 2002, Vokes et al., 2008).

In contrast, a *Shh* gain of function phenotype was detected in *Tg373^{orpk}* (Ohazama et al., 2009). Ectopic tooth development was detected next to the first molar as a result of ectopic *Shh* expression in the diastema. In these mice, *Ptc-1* and *Gli-1* were up-regulated in response to *Shh* signal transduction in the molar and incisor regions and expanded to the diastema. *Polaris^{fl/fl};CreERTm* mice did not show any abnormalities in tooth number, this might be due to different onset timing of *Polaris* gene deletion (Metzger and Chambon, 2001). Inhibition of hedgehog signalling using *HhAntag* for 28 days disrupts a generation of ameloblasts from stem cells in adult mouse incisor (Seidel et al., 2010b). Histological analysis of those mice showed the absence of proximal enamel in *HhAntag* treated animals and revealed severe morphological alterations as well as abnormal dentin formation. In *Polaris^{fl/fl};Cre ERTm* mouse incisors, ameloblasts and odontoblasts showed normal histology and polarization, suggesting that the *Shh* down-regulation in these mice was not low enough to induce any changes in mouse incisors or *Shh* down-regulation was compensated by other signalling pathways.

In summary, in *Polaris^{fl/fl};CreERTm*, *Thy-1* is down-regulated in the mouse mandibular incisor dental pulp. Nevertheless, incisor length and histology were maintained between the experimental group and a control group.

Chapter 6 : General Discussion and Future Consideration

Dental pulp stem cells are heterogeneous cell populations and their identities are not yet known because of the lack of specific markers (Bianco et al., 2008). Thy-1, is a GPI linked membrane protein, shown to be expressed in some stem and progenitor cells (Kon et al., 2009, Nakamura et al., 2006, Stevenson et al., 2009). *In vitro* and *in vivo* studies described in this thesis demonstrate that Thy-1⁺ population inside the mouse incisor dental pulp harbour stem cells and they are regulated by Shh signalling. However, it is also notable from our *in vivo* lineage tracing results that not all dental pulp cells and odontoblasts were generated from Thy-1⁺ cells. So, Thy-1 expressing population is not the only source of stem cells in the mouse incisor dental pulp.

In mouse incisor, the utilisation of transgenic reporter mouse lines enabled *in vivo* identification of two distinct mesenchymal cell populations that show potential dental pulp stem cell properties *in vivo*; 1) pericytes in the perivascular niche essential for vasculature homeostasis and 2) PRC1 (Polycomb repressive Complex 1) associated stem/progenitor cells in the apical area of the incisor (Feng et al., 2011).

Our results of *in situ* hybridization studies of stem cell genes, showed the expression of genes selected from a previous stem cell microarray in the apical area of the incisor. However, Thy-1 was the only investigated gene to be up-regulated in both incisors and the cervical loop areas which contain stem cell niches (Harada et al., 1999). Our *in vitro* studies also showed subpopulations of mouse incisor dental pulp

cells expressing *Thy-1* together with other mesenchymal stem cell markers as CD146 and CD133. However, their expressions declined in culture. This prompted us to study *Thy-1* expression *in vivo* using transgenic reporter mice *Thy-1Cre;R26R*, in which Cre expression is driven by the *Thy-1* promoter. Only cells that expressed or are expressing *Thy-1* are labelled. By tracing labelled cells in these mice, we showed that, although they were limited in number, they contributed to both incisor and molar tooth formation during the early stages of development. However, in adult stages, only mouse incisors are growing. Therefore, LacZ^+ cells in adult mouse incisor are derived from mesenchymal stem cells located at the cervical end of the incisor and any cells from early stages of differentiation will be lost during continuous growth. Since molars do not grow in adults, all LacZ^+ cells are developmentally derived. LacZ^+ cells were presented by odontoblasts and blood vessel walls. It is important to point out that DPSCs form odontoblasts *in vivo* (Gronthos et al., 2002) and the perivascular area is a well known stem cell niche (Shi and Gronthos, 2003). All together, these results indicate that the *Thy-1*⁺ population may contain a subpopulation of stem cells. However, not all odontoblasts were labelled with LacZ staining which denotes a complex MSCs niche.

Shh is the principle hedgehog gene in adult mouse incisors and responsive cells to *Shh* are stem cells inside the incisor (Seidel et al., 2010b). Using transgenic mice, *Thy-1* expression was regulated *in vivo* by *Shh* in adult mouse incisor. Its expression was increased in *Ptc-1*^{fl/fl};*CreER*^{Tm+} mice where *Shh* signalling increases and was decreased in *Polaris*^{fl/fl};*CreER*^{Tm+} mice where *Shh* signalling decreased. Therefore, we assumed that *Thy-1* is a marker for a stem cell subpopulation in mouse incisor dental pulp and it is regulated by *Shh*.

Dental pulps were selected for this study as, 1) it is easy to access and collect these cells and there is a very low morbidity after the extraction of the dental pulp (Gronthos et al., 2000a, Miura et al., 2003, Gronthos et al., 2002, Nakamura et al., 2009, Arora et al., 2009) ; 2) autologous DPSCs can be efficiently isolated and amplified from an impacted molar or exfoliated deciduous tooth (Arora et al., 2009, Nakamura et al., 2009, Gronthos et al., 2000a, Miura et al., 2003, Yu et al., 2007b); 3) DPSCs can generate much more typical dentin tissue within a short period than non-dental stem cells, which makes them more competent in making a bio-tooth (Yu et al., 2007b); 4) DPSCs can be safely cryopreserved and recombined with many scaffolds (Ding et al., 2010a, Gronthos et al., 2000a); and finally 5) DPSCs seem to possess immuno-privilege and anti-inflammatory abilities favourable for allotransplantation experiments (Graziano et al., 2008).

Thy-1 was selected for further study in this project because 1) it is a well known mesenchymal stem cell marker (Horwitz, et al. 2005). 2) It is expressed in many stem cells in other tissues (Nakamura, et al. 2006; Stevenson, et al. 2009). 3) Previous stem cells microarray results showed that it was up-regulated in the mouse incisor. 4) Our body against cervical loop microarray results revealed that it was up-regulated in the cervical loop area. 5) Its expression by *in situ* hybridization was more intense than the expression of the other genes found to be up-regulated in incisors, and finally 6) Thy-1 expression in mouse incisor dental pulp has not been studied before.

Mouse incisors were selected as a model of study because of their continuous growth resulting from the presence of stem cells in the cervical loop area (Harada et al.,

1999). Mandibular mouse incisors were preferred to upper ones because they are easier to extract. The age of 5 days was selected because teeth are easy to extract, permeable and fast to decalcify, and this age is similar to the one selected for microarrays samples.

6.1 Thy-1 in mouse dental pulp *in vitro*

MSCs represent a rare population in tissues. Therefore, it is essential to grow them *in vitro* before putting them into therapeutic use.

Thy-1 positive cells derived from PN5 dental pulp declined with cell passaging. This indicates that Thy-1⁺ populations are not able to be maintained in high numbers, at least using average culture conditions. Also, in the same cultures, double staining of Thy-1 with other mesenchymal stem cell markers (CD146 and CD133) revealed many subpopulations. These subpopulations behave differently in culture. Nevertheless, they were all substantially decreased by passage 5. This matches with the finding that in passage 5 of expanded tooth dental pulp, CD146⁺, Stro-1⁺ and CD133⁺ expression were decreased (Yang et al., 2010). However, this decrease in stem cell markers expression could be due to the fact that average culture conditions cannot completely simulate the *in situ* niches of stem cells (Ema, et al. 2000). Additionally, this could be due to Thy-1⁺ cell multi-differentiation ability, which could be because of some growth factors in the medium or serum which may induce their spontaneous differentiation *in vitro* (d'Aquino, et al. 2007).

Optimizing the cell culture conditions to support Thy-1⁺, CD146⁺ and CD133⁺ proliferation and expansion is recommended. Many studies were performed to test the optimal conditions to maintain MSC in the undifferentiated phenotype, starting

from usage of different media and sera concentrations (Lapi et al., 2008, Ayatollahi et al., 2012, Chase et al., 2010) or serum free media (Chase et al., 2010). However, these studies suggested that, different progenitor cells have differential sensitivity to media, sera and growth factors. Accordingly, the choice of culture media, supplements and serum compositions have to be carefully considered. Recently, a culture of human mesenchymal stem cells at low oxygen tension improved growth and genetic stability by activating glycolysis and reducing oxidative phosphorylation (Estrada et al., 2012).

CD146⁺ and CD133⁺ populations that had been previously studied (Schwab et al., 2008, Sorrentino et al., 2008, Shmelkov et al., 2008). CD133⁺/Thy-1⁺ double positive populations, were suggested as being an undifferentiated potentially multipotent cell population in human dental pulp (d'Aquino et al., 2007a). It has also been suggested that other populations that show positivity for CD146⁺ and Thy-1⁺ with other markers represent a subpopulation of stromal vascular progenitors in adult human adipose tissue (Zimmerlin et al., 2010). Further investigation such as isolation and *in vitro* differentiation are necessary to explore the stem cell properties of these double positive populations inside the mouse dental pulp.

Thy-1⁺ expression level also decreased in sorted cells. This could be due to culture conditions as discussed before or due to the imbalance between Thy-1⁺ and Thy-1⁻ cell populations. In stem cell niches, contact between stem and non-stem cells keeps the former in an undifferentiated status, thus, loss of this contact leads to stem cells differentiation. Similar to the *in vivo* stem cell niche, Thy-1⁻ cells contact with Thy-1⁺ cells may help to maintain their undifferentiated status. Loss of this contact may lead to loss of their Thy-1 expression, possibly due to their differentiation. Also, this

significant decrease in Thy-1 expression level could be due to cell damage as a result of using MACS for sorting. In a study comparing the two methods, MACS and FACS for mouse CD4⁺ CD25⁺ regulatory T cells, isolation showed that magnetic cell sorting took more time and effort which led to fewer live cells obtained when compared with flow cytometry sorting (Yan et al., 2009). However, these methods are dependent on the existence of specific cell surface antigens and the formulation/availability of high affinity probes to these antigens. An irreversible attachment of these probes to target cells also has the potential to influence cell behaviour. In the absence of a specific or unique marker or to avoid potentially confounding interactions of probes with cells, and to facilitate achieving the objectives of scalability and non-invasiveness mentioned above, dielectrophoresis (DEP) was recently used to sort cells based on the premise that different types of cells have different electric properties (Pethig et al., 2010). So this technique could be used for sorting Thy-1 cells if Thy-1⁺ electric properties are different from Thy-1⁻ ones, but further studies are necessary to explore that.

In this study, osteogenic induction for dental pulp cells at passage 2 and 4 showed osteogenic differentiation in both passages. But, osteogenic layers were formed in passage 2 while osteogenic patches were formed in passage 4. Interestingly, Thy-1 expression level was directly proportional with the level of osteogenic potentiality. This may suggest Thy-1 expression level as an indicator for osteogenic differentiation in a cell population. However, this could be confirmed by comparing the osteogenic differentiation between Thy-1⁺ and Thy-1⁻ populations. Also, these Thy-1⁺ populations can be selected and inserted into an artificial bony defect to investigate their osteogenic potentiality *in vivo*.

However, other mesenchymal stem cell markers as CD133 and CD146 were also declining in culture. So, this reduction in osteogenic potentiality could be as a result of decreased stem cells in culture. Investigating the differentiation to adipogenic and chondrogenic lineages will declare if decreased osteogenic potentiality is due to Thy-1 expression declining or due to reduced stem cell populations in cell culture.

6.2 Thy-1 in mouse dental pulp *in vivo*

Previously, the multilineage differentiation potential of adult stem cells was demonstrated by incubating such cells in culture media with specific additives and subsequently identifying lineage-specific changes according to morphological and phenotypical criteria (Zhang et al., 2006). However, the *in vitro* and *in vivo* situations are unquestionably different environments. In addition, RT-PCR or immuno-methods used in these studies to monitor the changes of cell phenotype, can only distinguish the expression of specific markers but are unable to prove functional changes. For these reasons, studying stem cells *in vivo* reveals their true properties.

In this study, using *in situ* hybridization to investigate Thy-1 expression at three different stages showed that its expression was restricted to the cervical loop area of the mouse incisor dental pulp which houses the mesenchymal stem cell niche (Feng, et al. 2011). This shows that *Thy-1* expressing cells may contain a stem cell population inside mouse incisor dental pulp, as true stem cells persist throughout life because of their unlimited self-renewal. This agrees with the study in which *Thy-1* was expressed in hBMSCs from donors of different ages although it was significantly reduced with age (Stolzing, et al. 2008). Further experiments are needed to investigate the expression of other mesenchymal stem cell markers as CD133 and CD146 in adult mouse incisor. Another study using *in situ* hybridization showed a

population of *Bcor* expressing cells resident in the apical mesenchyme of mouse incisor (Lapthanasupkul et al., 2012) at the same site for *Thy-1*. These populations were highly proliferative and were able to migrate to the injury site (Feng, et al. 2011). Further studies are needed to explore the relationship between *Bcor*⁺ and *Thy-1*⁺ populations.

In vivo lineage tracing, using the Cre-loxP recombination system, has been used successfully to determine cell fate by following the progeny of labelled cells through the differentiation process (Zhang et al., 2008). In *Thy-1Cre;R26R*, *in vivo* tracing of *LacZ*⁺ cells showed their small contribution to tooth formation during the pre-natal stage of tooth development. Nevertheless, they contributed more to dental pulp cells including odontoblasts, during the post-natal and adult stages. The presence of these *LacZ*⁺ cells in the dental papilla during tooth development suggests their neural crest origin. Investigating the expression of neural crest cell markers in pre-natal and postnatal stages is required to check if they express/maintain their neural crest origin from early development to adulthood or not?

During adult stages, because mouse incisors grow continuously in contrast to mouse molar, so, *LacZ*⁺ cells in adult mouse incisor came from *Thy-1*⁺ stem/progenitor cells at the cervical end.

Most markers that are active in stem cells are not exclusive to these cells, resulting in a heterogeneous group of stem cells and early progeny that are marked (Fuchs and Horsley, 2011). *Thy-1* is found on a variety of cell types including thymocytes, peripheral T cells, myoblasts, epidermal cells, and keratinocytes (Pont 1987; Reif and Allen 1964).

In Thy-1Cre^{+/+};R26R^{+/+} mouse dental pulp, many cell lineages such as odontoblasts, pericytes/endothelial cells and pulp cells and probably fibroblasts were all LacZ⁺. Identity of odontoblasts could be confirmed through immunostaining with one of the odontoblast markers such as Nestin (Ogawa, et al. 2006). Some LacZ⁺ cells were correlated to blood vessel walls from the early stage of tooth development until the adult stage. These cells could be pericytes that were previously described as mesenchymal stem cells inside mouse dental pulp (Feng, et al. 2011; Zhao, et al. 2012) or could be endothelial cells. Immunostaining with pericytes/endothelial markers such as NG2, 3G5 (for pericytes) CD105, CD31 and Von Willebrand factor (for endothelial cells) will declare the nature of these cells. This shows the complexity of Thy-1 population inside the mouse dental pulp, this could be revealed by using Brainbow mice (Weissman et al., 2011).

The Brainbow approach is designed to label cells with distinct fluorescent proteins (FPs), such as cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), or red fluorescent protein (RFP), using a single transgene (Livet et al., 2007, Lichtman et al., 2008). On the basis of Cre/lox-mediated DNA excision or inversion (Branda and Dymecki, 2004), brainbow transgenes trigger the stochastic expression of two to four XFPs in a cellular population, such that each cell randomly adopts one color. If only one copy of the Brainbow construct is present in cells, the recombination choice leads to mutually exclusive expression of XFPs. For example, cell A will express one XFP, whereas cell B will express a different XFP. However, when multiple Brainbow transgene copies coexist in cells, each copy makes its own stochastic choice, yielding combinatorial expression of XFPs and creating a wide range of additional colours. In other words, cell A may express one or a combination of XFPs, whereas cell B may express a different combination or ratio of XFP. In this

way, each Thy-1⁺ cell and its daughters will show the same color, so their tracking will show which population goes to odontoblasts and which goes for blood vessel walls or if they both contribute to each other.

In Thy-1Cre^{+/-}; R26R^{+/-}, LacZ⁺ cells are not necessarily expressing Thy-1 at the time of investigation. An inducible Cre system will label cells expressing Thy-1 only at the time of induction and this will help to identify the newly generated odontoblasts which in turn will reveal the nature of the rhythmic pattern of LacZ⁺ odontoblasts formation.

6.2.1 Regulation of Thy-1 expression in mouse incisor

Stem cells play a critical role in tissue homeostasis and repair throughout life. Their fate between self-renewal and differentiation is regulated by both cell intrinsic determinants and signals from a specialized microenvironment (Moore and Lemischka, 2006).

The growing interest in the molecular regulation of stem cells arises from the potential to influence their fate and consequently their functions during tissue repair and/or regeneration (Mitsiadis and Rahiotis, 2004). It has been proposed that Shh plays a number of roles in stem cell biology, including regulation of fate decisions in embryonic stem cells (Gaspard et al., 2008) and survival and self-renewal of neural stem cells (Machold et al., 2003, Balordi and Fishell, 2007).

In PN5 wild type mouse incisor organ culture and adult Ptc-1^{fl/fl}; CreERTm mouse incisor, Thy-1 expression was up-regulated with increased Shh signalling. However, Thy-1⁺ cells were not responsive to Shh in a wild type cell culture, which may indicate their need to an intact epithelial-mesenchymal contact or other factors in the *in vivo* microenvironment in order to respond to Shh signalling. X-ray CT scans

showed that there was no significant difference in mouse incisors length between Tam treated and control groups, Also, ameloblasts and odontoblasts showed normal histology in both groups.

In *Polaris^{fl/fl};CreERTm* mice, Thy-1 expression was down-regulated with Shh signalling repression. This repression in Shh signalling is in contrast with its increase in E13.5 *Tg737orpk* which showed an increase in *Ptc-1* and *Gli-1* expression and decreased in Shh antagonist *Gas 1* expression denoting Shh increased signalling (Ohazama et al., 2009). Accordingly *Tg737orpk* adult mice had teeth mesial to first molar in all four jaw quadrants with 100% penetrance that were not present in wild-type animals (Ohazama et al., 2009). This contrasts with *Polaris^{fl/fl};CreERTm* adult mice which had normal histology and equal incisor length to the control group. This contrast in Shh signalling and its consequences could be due to different onset timing of *Polaris* gene deletion. Investigating Thy-1 expression in an early developmental stage of *Tg737orpk* will explore if it has a role in extra tooth formation.

Inhibition of hedgehog signalling disrupts generations of ameloblasts from stem cells (Seidel et al., 2010b). Thy-1⁺ progeny cells contribute to odontoblast formation in mouse incisor (from our results in Thy-1 *Cre^{+/-};R26R^{+/-}* mice). The presence of normal ameloblasts and odontoblasts in *Polaris^{fl/fl};CreERTm* adult mice in spite of Shh down regulation, contradicts the study in which adult mice showed disruption in ameloblasts generation, absence of proximal enamel and revealed severe morphological alterations as well as abnormal dentin formation after receiving a Hh antagonist for 28 days (Seidel et al., 2010b). This could be explained by saying that

Shh down-regulation in Tam treated mice was not low enough to induce these changes.

In summary, the work described in this thesis demonstrated that; 1) Thy-1 was expressed in mouse incisor dental pulp cells during different stages of mouse tooth development. 2) Thy-1⁺ cells harboured a sub population of hard tissue forming stem cells contributed for mouse incisor continuous growth. 3) Thy-1 was regulated by Shh signalling in adult mouse incisor. By applying this to human teeth, Thy-1⁺ sub population could be used to regenerate dentin/bone in dentinal/periodontal defects respectively.

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6.3.1 Microarray

Affymetrix, Inc.

The R statistics for Statistical Computing and graphics

(v.2.13.2)

Subcloning Efficiency™ DH5α™ Competent cells

1% Tryptone

OXOID Ltd., LP0042

1% NaCl

BDH, 102415K

0.5% Yeast

OXOID Ltd., LP0021

Luria-Bertani (LB) agar

1% Tryptone

OXOID Ltd., LP0042

1% NaCl

BDH, 102415K

0.5% Yeast

OXOID Ltd., LP0042

1.5% Agar

OXOID Ltd., LP0011

Ampicillin Sodium Salts (50 mg/ml)

SIGMA, A9518

Fast Plasmid® Mini

Eppendorf AG, 955150601

QIAGEN Plasmid Maxi Kit

QIAGEN 12163

QIAquick® Gel Extraction Kit

QIAGEN 28706

Restriction enzymes and buffers

Promega

Paraformaldehyde

(4% Stock solution in Nuclease-free 1XPBS)

SIGMA, P6148

Diethyl pyrocarbonate (DEPC)

SIGMA, D5758

Formic acid, 98%

BDH, 20320.320

Ethylenediaminetetraacetic acid (EDTA)

VWR, 20302.293

Ethanol

VWR, 101077Y

1,2,3,4-Tetrahydronaphthalene

SIGMA, 429325

Histoclear

National Diagnostics.

HS-202

Ultraplast Polyisobutylene Histological Wax	Solmedia, WAX060
Erhlich's Haematoxylin	Solemedia, HST003
Eosin, aqueous solution	
(0.5% Eosin Y in distilled H ₂ O)	Riedel-de Haën, 32617
DePex	BDH, 360294H

6.3.4 *In-situ* hybridization (ISH)**6.3.5 List of plasmids (See Table 2-1)****Table 0-1: Details for plasmids used for making anti-sense probe**

Gene	Vector	Size of insert	Digestion Enzyme to Linearise Plasmid DNA	Polymerase Enzyme to Generate Antisense Probe
Crabp1	PT7T3D-Pac1	1.3Kb	Sfi1	T3
Hus-1	pSport1	2kb	Kpn1	Sp6
Thy-1	pCMV-SPORT6	2.3kb	Kpn1	T7
Ikros1	PT7T3D-Pac1	1.5kb	Pac1	T7
Shh	pBluescript	2.6 kb	EcoR1	T7
Gli-1	Bluescript	1.7 kb	Not1	T3
Ptc-1	pBluescript	1 kb	BamH1	T3

6.3.6 Solutions and reagents

Polymerase enzymes	Promega
DIG RNA labelling Mix (10X)	Roche, 11277073910
SigmaSpin™Post-Reaction Clean-Up Column	Sigma, 5059
DL-Dithiothreitol (DTT)	MP Biomedicals, 100597
Triton® X-100	
(Iso-Octylphenoxypolyethoxyethanol)	BDH, 306324N
Tween-20	Sigma, P7949
IGEPAL CA-630	Sigma, I3021
Proteinase K	Sigma, P2308
Glycine	Sigma, G7403
Formamide	Merck, K36952408
tRNA (RNA from yeast)	Roche, 109223
Blocking Reagent	Roche, 11096176001

Heparin lithium salt	
(From Porcine Interstinal mucosa)	Sigma, H08078
SDS (Sodium dodecyl sulfate)	Severn, 30-33-50
Anti-Digoxigenin-AP Fab fragments	Roche, 11093274910
NBT (4-Nitro blue tetrazolium chloride)	Roche, 11383213001
BCIP (5-Bromo-4-chloro-3-indolyl-phosphate)	Roche, 11383221001
Polyvinyl alcohol	BDH, 297914D
TEA (Triethanolamine)	BDH, 103704U
Acetic anhydride	BDH, 100022M
50x Denhardt's	
1% (w/v) Ficoll 400	Sigma, F4375
1% (w/v) Polyvinylpyrrolidone	BDH, 436032C
1% (w/v) Bovine Serum Albumin	Sigma, A9647
50% Dextran sulphate	Chemicon, 0702051849
6.3.7 Organ Culture	
Organ Culture Dish	
(Center-Well Organ Culture Dish)	Falcon®353037
MF-Millipore Membrane, mixed cellulose esters	
(0.1 µm White VCWP 25 mm)	Millipore, VCWP02500
Alpha MEM Eagle w/ UGln1 and nucleosides	Lonza- BE02-002F
L-Glutamine	SIGMA, G7513
Fetal Bovine Serum (FBS)	Lonza, DE14-801F
Penicillin-Streptomycin solution	SIGMA, P0781
DMSO (Dimethyl sulfoxide)	SIGMA, D-8779
Cyclopamine	Toronto Research, Chemicals, C988400
Smoothened Agonist, SAG	Merck, 566660-1MG
6.3.8 Staining for β-galactosidase (LacZ) Activity	
Trizma® base (Tris base)	Sigma, T1503
Glutaraldehyde	Merck, 1042390250
Sodium deoxycholate	Sigma, D6750

IGEPAL CA-630 (NP-40)	Sigma, I3021
Potassium ferrocyanide ($K_4[Fe(CN)_6]$)	BDH, 102054F
Potassium ferricyanide ($K_3[Fe(CN)_6]$)	BDH, 102044D
Magnesium chloride ($MgCl_2$)	Fisher, BP214-500
X-Gal	Fermentas, R0404
5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside	
Phosphate buffered saline (PBS)	Fisher, BP-665-1
Methanol	Fisher, M/4056/PB17
Propan-2-ol (isopropanol)	Acors Organics, 389710025
1,2,3,4 -Tetrahydronaphthalene	Sigma, 429325
Eosin, Alcoholic Solution (in Ethanol)	
0.25% Eosin Y disodium salt	Riedel-de Haën, 32617
21% Distilled H_2O	
Nuclear Fast Red (in H_2O)	
0.2% Nuclear Fast Red	Sigma, 60700
O.C.T. compound	BDH (361306E)
Sucrose	Sigma (S0389)

6.3.9 Cell culture

Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma, D1408
Trypsin-EDTA solution	Sigma, T4049

6.3.10 Flow cytometry

6.3.10.1 FACS blocking buffer

Fetal Bovine Serum (FBS)	Lonza, DE14-801F
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6.3.10.2 Antibodies (See Table 2-2)

Table 0-2: Antibodies and their optimal dilution used in flow cytometry

Gene	Dilution	Gene Localization	Manufacture
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Mouse monoclonal to Thy-1 CD 90 conjugated to Fluorescein Isothiocyanate (FITC)	1:100	Cell Surface	Abcam 62009
Mouse monoclonal [P1H12] to CD146 conjugated to Phycoerythrin (PE)	1:70	Cell surface	Abcam 75769
Rat anti mouse monoclonal to CD133 (Prominin-1) conjugated with Allophycocyanin (APC)	1:700	Cell surface	e- bioscience
Purified rabbit polyclonal to Patched / PTC	1:50	Cell surface	Abcam 53715
Mouse IgG2bk isotype control FITC	1:100	Cell surface	e-bioscience

6.3.11 Magnetic Activated Cell Sorting (MACS)

Anti-FITC MicroBeads
Miltenyi Biotec,
130-048-701

MACS Buffer (0.5% BSA ,2mM EDTA in PBS)

Albumin, from bovine serum (BSA) Sigma, A9418

6.3.12 Optical Projection Tomography (OPT)

Agarose, low gelling temperature Sigma, A9414-100G

Folded filter papers Whatman

Benzyle Benzoate Sigma, W213810

Magnetic moulds Bioptonic

Bioptonic scanner 3001 Bioptonic

6.3.13 In vitro differentiation

StemPro® Osteogenesis Differentiation Kit Gibco®, A10071-01

Alizarin Red S Sigma, A5533

6.3.14 Immunohistochemistry

6.3.14.1 Antibodies (See Table 2-3)

Table 0-3: Antibodies and their optimal dilution used in immunohistochemistry

Gene	Dilution	Gene location	manufacture
Purified Rabbit monoclonal to CD146	1:200	Cell surface	Abcam, ab75769
Purified Rabbit polyclonal to CD133	1:100	Cell surface	Abcam,ab19898
Purified rabbit polyclonal to Thy-1	1:100	Cell surface	Sc-9163
Biotinylated goat anti-rabbit IgG	1:200	Cell surface	Vector,BA-1000

6.3.14.2 Other Reagents and Solutions**Blocking Buffer (in 1XPBS)**

1% Albumin from bovine serum

Sigma, A4919

10% FBS

Sigma, F7524

Citric Acid

Sigma, C7129

Vectastain Elite ABC Kit

Vector Labs, PK-6101

DAB Peroxidase Substrate Kit

Vector, SK-4100

Hematoxylin solution according to Delafield
(Counterstain)

Fluka, 03971

VectaMount Permanent Mounting Medium

Vector, H-5000